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# Perfusion immunoassay for acetylcholinesterase: analyte detection based on intrinsic activity

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**Abstract**

A quantitative, two-column, HPLC-based assay requiring only 30 min to complete is reported. Amniotic fluid proteins are first fractionated on a size-exclusion column; the fraction containing the $M_r$ 280 000 neural acetylcholinesterase (AChE) is then diverted to a second column, an ImmunoDetection cartridge derivatized with an anti-AChE antibody. The immobilized antibody traps the enzyme, then substrate is flowed through the cartridge and the product is detected. For a positive result, the enzyme must have a molecular mass corresponding to the neural-AChE, be recognized by the antibody and be active in converting the substrate into product. The assay is sensitive in the clinically relevant range. The method provides rapid quantitative analysis using an automated instrument projected to be suitable for screening large numbers of samples.

## 1. Introduction

This paper reports the exploration of several HPLC-based methods to improve and automate the determination of (AChE) acetylcholinesterase in amniotic fluid. A full clinical evaluation of these methods will be presented separately. Clinically useful analytical methods for AChE (EC 3.1.1.7) must account for the fact that there are several proteins with related enzymatic activity [1,2]. AChE is present in neural tissue as a tetramer (G4 form) with a relative molecular mass of about 280 000. It is also present in erythrocyte membranes as a dimer (G2 form) with approximately half the molecular mass. Soluble forms of AChE can be found in amniotic fluid coming from improperly formed neural tubes or ventral defects [3], or on rare occasions from fetal serum, which also contains AChE activity, and may be introduced during amniocentesis [4]. AChE has a substrate preference for acetylcholine and is inhibited by BW284C51. In contrast, butyrylcholinesterase (BuChE) (EC 3.1.1.8), also called "pseudocholinesterase", has a slightly different substrate selectivity and is resistant to inhibition by BW284C51. BuChE is relatively ubiquitous in serum and amniotic fluid, although at varying levels. Although BuChE has the same molecular mass as AChE, it is both antigenically and electrophoretically distinct [5,6].

An elevated AChE level in amniotic fluid is a clinically relevant marker of neutral tube defects (NTD) in developing human fetuses [3–5]. As currently practised, screening for NTD usually begins by screening maternal serum for $\alpha$-fetoprotein (AFP). Patients with elevated maternal serum AFP have typically 10–20 ml of

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amniotic fluid drawn for analysis and an ultrasound scan. The amniotic fluid is tested for AFP and AChE. Literature reports give the normal level of neural AChE in amniotic fluid as ca. 0.002 IU/ml (corresponding to ca. 2 ng/ml, based on an estimated specific activity of 10 000 IU/mg). Abnormal levels of AChE in amniotic fluid are ca. 0.01 IU/ml or 10 ng/ml using the same estimate of specific activity. The currently used gel AChE assay uses non-denaturing polyacrylamide gel electrophoresis to separate AChE (fast migrating) from BuChE (slow migrating). Enzyme activity is detected *in situ* in the gel by incubating it with acetylthiocholine iodide substrate, which releases a free thiol on cleavage. The resulting thiol may be detected with either dithiooxamide or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), both of which give a yellow colour. Samples with elevated AChE have the assay repeated with the inhibitor BW284C51 included with the substrate to show selective inhibition. Samples with elevated AChE may be strongly positive (associated with NTD) or weakly positive (associated with ventral wall defects) [7]. Detection of weakly positive samples can be difficult, and reflects the fact that the assay sensitivity is at the low end of clinically relevant levels. False-positive results can occur if excessive fetal serum-derived AChE is present in the amniotic fluid, so in positive samples a test for fetal hemoglobin (Hb) is also run for evidence of unusually high fetal blood contamination.

The gel-based AChE diagnostic assay suffers from several limitations. Gel electrophoresis and staining are slow and not automated. It takes 2 days until the results for the first gel are available, and another 2 days to run a confirmatory gel for the enzyme inhibition assay. The gels are limited in sample volume to 30 $\mu$l of amniotic fluid, whereas up to 20 ml of sample volume are potentially available. As the detection of weakly positive samples is important, improved assay sensitivity is desirable, and might be achieved if more of the available sample could be used.

While antibodies can distinguish BuChE from AChE, and some antibodies are reported to show some selective preference for G4 over G2 [8], immunochemical procedures are not compatible with the gel format. Finally, *in situ* staining for enzyme activity is only semi-quantitative, while there is the possibility that a more quantitative assay might facilitate differential diagnosis of an underlying developmental defect [9].

This paper reports the development of a combined analytical method, involving automated separation of G2 and G4 AChE by size-exclusion chromatography (SEC), followed by capture of the G4 AChE with an anti-AChE antibody immobilized on an ImmunoDetection (ID) cartridge [10].

## 2. Experimental

### 2.1. Antibodies

Two anti-AChE antibodies were evaluated: 4F19 (DAKO, Capenteria, CA, USA) and AE-1 (hybridoma obtained from American Type Culture Collection, Rockville, MD, USA) [7]. The AE-1 hybridoma was grown at the Genetics & IVF Institute (Fairfax, VA, USA) and the antibody was purified by protein G chromatography on BioCAD (PerSeptive Biosystems, Framingham, MA, USA). Both antibodies were biotinylated with NHS-LC-Biotin (Pierce, Rockford, IL, USA).

### 2.2. Enzyme substrate

The substrate used was acetylthiocholine iodide. Enzyme action on this substrate produces a free thiol, which is detected by reaction with DTNB to form a coloured product that is monitored by absorbtion at 420 nm. The substrate solution consisted of 100 mM phosphate buffer (pH 6.9) with 1 m*M* acetylthiocholine and 33 $\mu$g/ml DTNB. In some instances inhibitor BW284C51 was added to the substrate solution at a 50 $\mu$M concentration. Unless specified otherwise, all reagents were purchased from Sigma (St. Louis, MO, USA).

### 2.3. Samples and standards

Two analytical standards were used to estimate the molecular masses of proteins on the size-exclusion chromatogram: goat IgG ($M_r$

150 000) (Jackson ImmunoResearch Labs., West Grove, PA, USA) and bovine hemoglobin ($M_r$ 60 000). Several sources of AChE were analyzed, including purified electric eel AChE and semi-pure human erythrocyte AChE. Samples of human amniotic fluid were provided by the Genetics and IVF Institute. These were amniotic fluid samples previously identified as normal, or significantly positive for AChE, based on the gel AChE assay, and a sample from fetal demise.

### 2.4. Columns

The SEC column was 200 × 9.4 mm I.D. polyhydroxyethyl A of 5 μm particle size (PolyLC, Columbia, MD, USA). Two different ID cartridge formats were evaluated. Both contained 20 μm POROS streptavidin media (PerSeptive Biosystems, Framingham, MA, USA) on which biotinylated anti-AChE antibody had been immobilized. In one instance the cartridge had a 16-μl volume (20 × 1 mm I.D.) and 150 μg of antibody ("mini-ID") and in the other the cartridge had a 100-μl volume (30 × 2.1 mm I.D.) and 1 mg of antibody (standard ID). When the mini-ID cartridge was used the reagent loop was 200 μl, whereas when the standard ID cartridge was used the reagent loop was 2 ml.

### 2.5. Buffers

The loading buffer for SEC and for direct injection on to the ID cartridge was 10 m*M* phosphate-buffered saline (PBS) (pH 7.0). The elution buffer for regenerating the ID cartridge was 12 m*M* HCl (pH 2.0).

### 2.6. Chromatography

Two forms of assay were developed. The short form uses only the ID cartridge with immobilized antibody to AChE. Sample is injected directly into the ID cartridge and the total AChE activity is determined. This short assay is used to screen samples for elevated AChE. The long form of the assay is a two-column assay, with the sample injected into the size-exclusion column for SEC followed by diverting the appropriately sized proteins to the ID cartridge. The short form of the assay may be sufficient for clinical applications using amniotic fluid, where the low solubility of the red blood cell (RBC)-derived G2 form means it is unlikely to influence the assay. The long form of the assay allows the option of confirming the molecular mass of the antigen in test, as well as offering molecular and cell biologists the opportunity to measure separately both G2 and G4 AChE using the same antibody. Fig. 1 itemizes the individual steps in the long-form method. Fig. 2 shows the plumbing diagram for executing these steps.

The experiments were run on the INTEGRAL Micro-Analytical Workstation (PerSeptive Bio-

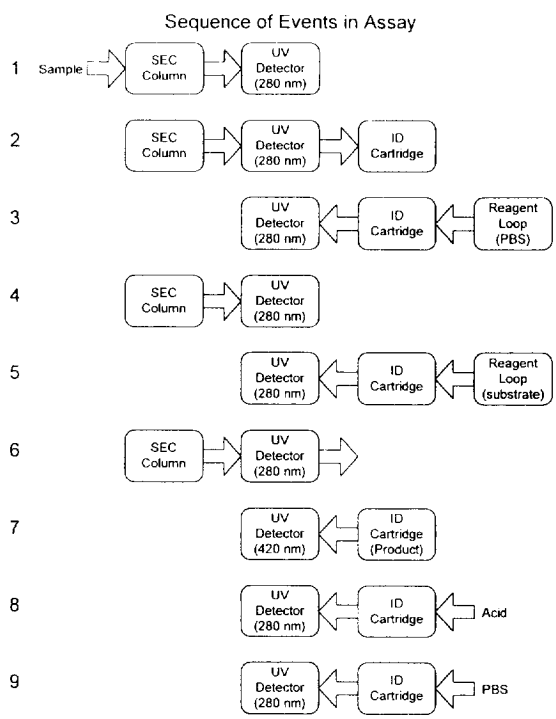


Fig. 1. Sequence of events in the assay. This chart illustrates the flow to the columns, detector and reagent loop during the course of the two-column assay. The individual steps are described in the text. The Integral system allows automated programming of the valve, pump and detector changes needed to execute these steps.

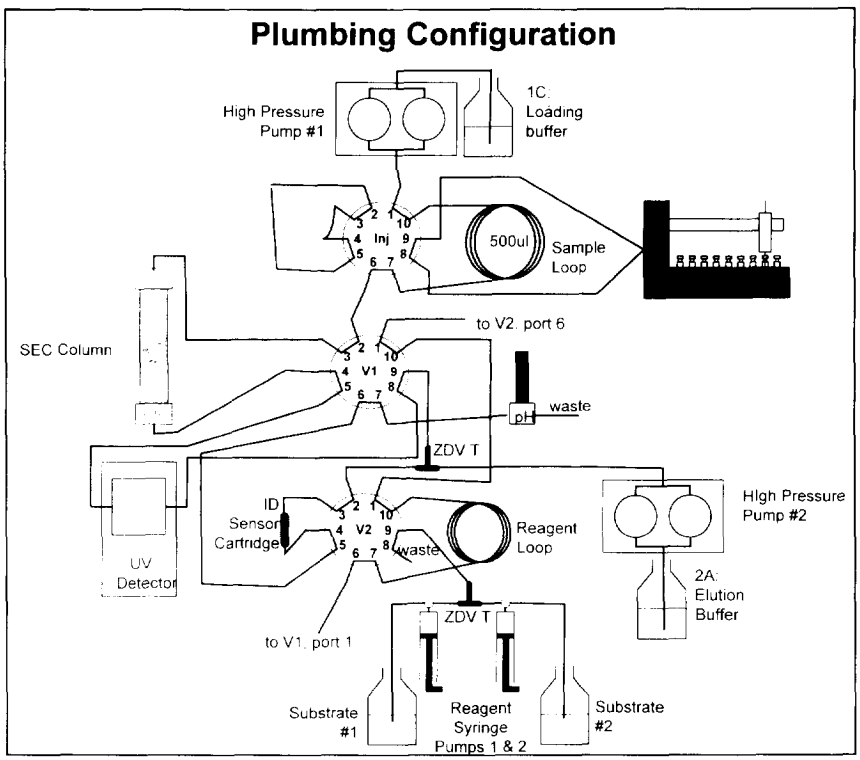


Fig. 2. Plumbing configuration. This figure illustrates the features of the Integral system used in this method. The autosampler delivers 200-$\mu$l samples of standards and amniotic fluid. Valve 1 has connected to it the SEC column and the UV detector. Valve 2 has connected to it the ID cartridge with the immobilized anti-AChE antibodies and a reagent loop. The loop may be filled with substrate solutions using either syringe pump 1 or 2. The syringes are linked with a zero-dead-volume T-piece (ZDV T). The second HPLC pump delivers elution buffer via a ZDV T connection just upstream of the ID cartridge. For the short form of the method, were no SEC is done, the SEC column was replaced with a union, but otherwise the plumbing configuration was unchanged. If the ID cartridge was of the standard 100-$\mu$l volume, the reagent loop was 2 ml. If the ID cartridge was of the 16-$\mu$l volume, then the reagent loop was 200 $\mu$l.

systems), which is equipped with a built-in autosampler, three ten-port valves, two HPLC pumps, two syringe pumps, a built-in UV detector and a pH probe. A computer method controls the sample injection, valve changes, reagent additions and data collection to execute the following steps.

*Step 1.* At the start of the method, a 200-$\mu$l sample is injected from the autosampler on to the size-exclusion column. Effluent is monitored by the UV detector, set at 280 nm.

*Step 2.* At a retention time corresponding approximately to that of G4 AChE ($M_r \approx$ 200 000–400 000), the ID cartridge is placed in-line after the UV detector by automatic valve rotation. The anti-AChE antibody on the ID cartridge captures the G4 AChE. The UV detector is situated between the two columns in the flow.

*Step 3.* The ID cartridge is washed to remove unbound material by putting the reagent loop, previously filled with PBS, in-line upstream. The SEC column is off-line during this step. The detector signal reflects the unbound proteins being washed from the cartridge, since the detector is now downstream from the ID cartridge.

*Step 4.* The ID cartridge is taken off-line while materials with lower molecular masses are

eluted from the SEC column. Also, during this time the reagent loop is filled with substrate solution from syringe pump 1. The SEC column is monitored by the UV detector.

*Step 5.* In this step the reagent loop, now filled with substrate, is again put in-line upstream from the ID cartridge, causing substrate to flow through the latter. A sufficient volume exists in the loop (200 μl for the 16 μl mini-ID cartridge, or 2 ml for the 100 μl standard ID cartridge) to insure that substrate completely flushes the cartridge and some flow-through is detected by the UV monitor downstream from the cartridge. DTNB has a large absorption at 280 nm and this presence of substrate in the detector confirms delivery of substrate reagent to the ID cartridge.

*Step 6.* The ID cartridge is taken off-line (4-min stopped-flow incubation period) and the immobilized AChE enzyme converts substrate into product. The ID cartridge is held at 37°C by submersion in a water-bath. During this incubation period the SEC column is washed and regenerated for use in the next sample. The UV detector monitors the SEC column effluent and confirms that it has returned to the baseline.

*Step 7.* At the start of this step the detector wavelength is shifted to 420 nm, corresponding to that of the DTNB-thiol choline product. The ID cartridge contains accumulated product formed by the action of the AChE on the substrate, and this product is pushed past the detector for quantification.

*Step 4′, 5′, 6′, 7′.* It is possible to repeat steps 4, 5, 6 and 7 with a second substrate delivered to the reagent loop from syringe pump 2. The syringe pumps are coupled through a zero-dead-volume T-piece and both access the reagent loop. This configuration is useful in developing methods by allowing direct comparison of substrate solutions, for example. In clinical applications this second substrate addition–incubation–detection sequence might allow comparison of a standard substrate in syringe 1 with a substrate plus enzyme inhibitor in syringe 2.

*Step 8.* Acid elution buffer from HPLC pump 2 elutes the bound AChE from the antibody on the ID cartridge. At this step the UV detector is again set to monitor at 280 nm, and the peak detected reflects the amount of protein desorbed from the ID cartridge.

*Step 9.* The ID cartridge is re-equilibrated with PBS from HPLC pump 1.

In the short form of the assay, the SEC column is replaced by a union and the plumbing configuration remains the same. Step 1 is eliminated, so the sample is injected through the UV detector into the ID cartridge in step 2. Step 4 is shortened to allow only time to fill the reagent loop with substrate. Step 6 is retained as a stopped-flow substrate incubation step, but washing of now unused SEC is not required.

## 3. Results

Fig. 3 shows the overall strategy used in this analysis. Materials separate in SEC on the basis of size, with the largest materials having the

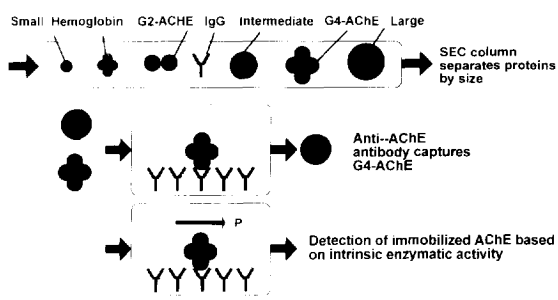


Fig. 3. Dual-column assay for AChE. The first column is a SEC column separating amniotic fluid components on the basis of their molecular size. Large proteins elute first followed by progressively smaller molecules. G4 AChE has a molecular mass of ca. 280 000, while G2-AChE is slightly smaller than γ-globulin at $M_r$ 140 000. Another common protein, hemoglobin ($M_r$ 60 000) has a longer retention time than G2 AChE. The fraction containing G4 AChE and similarly sized molecules is diverted to the antibody-coated ID cartridge as the second column. The antibody captures the AChE and unbound material is washed away. The captured AChE is detected based on its intrinsic enzymatic activity. Substrate flows on to the ID cartridge and is converted by the AChE into product. The ID cartridge is taken off-line and allowed to incubate for 4 min to allow product to accumulate. The product is then washed from the cartridge to the UV detector. As a final step in the method, the ID cartridge is washed with acid elution buffer and bound AChE is eluted.

shortest retention time. A fraction with the retention time of G4 AChE is diverted to the ID cartridge, where it is captured by an anti-AChE antibody. Final detection of the now immobilized AChE is effected by flowing the substrate into the cartridge and detecting the formation of product. The idea of using the enzymatic activity of AChE to enhance the detection of this antigen has been developed before [11]. To be positive in this assay, the analyte must have the correct size, epitope and enzymatic activity.

An example of a chromatogram obtained with the short form of the assay is illustrated in Fig. 4. In this case the 1 mm I.D. cartridge was used. The sample injection results in the first peak and the addition of the substrate causes the second peak. At this point the cartridge is taken off-line for the incubation period and the wavelength of the detector is changed from 280 to 420 nm. When flow resumes, a product peak is observed at about 9.5 min. In this example, a second, different, substrate was added from syringe pump 2 (corresponding to steps 5′, 6′ and 7′). The second substrate addition peak is not as large as the first, as the UV detector is now monitoring at 420 nm. The second incubation period results in a second product peak at 13.5 min. In the case illustrated, two different concentrations of DTNB were compared. For substrate 1 the DTNB concentration was 133 $\mu$g/ml, whereas in substrate 2 it was 33 $\mu$g/ml. The latter concentration was taken as the standard condition for later experiments, because although it gave a slightly smaller peak on a positive sample, the blank peak was reduced by a factor of four (data not shown). The pH trace in the chromatogram shows the final steps, namely acid elution and re-equilibration. During this elution step the UV monitor has been returned to 280 nm. A very small peak appears in the chromatogram which reflects both the valve change and a small amount of bound protein.

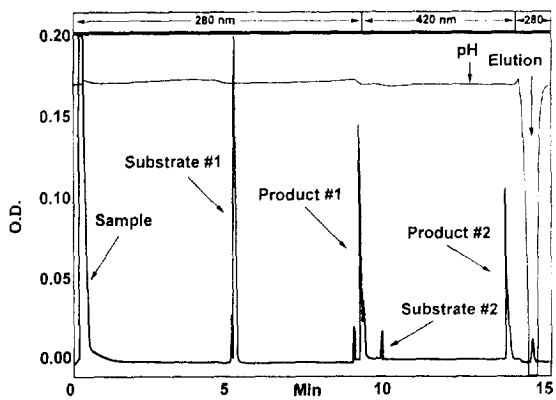


Fig. 4. AChE detection. This chromatogram illustrates the short form of the method, with the sample being injected directly on to the ID cartridge (no SEC column), and the use of two substrates. In this example, the 1 mm I.D. cartridge was used. Following washing of the column to remove unbound materials, the column is flooded with substrate 1 and taken off-line for 4 min. At about 9 min into the method the column is returned to the flow and product from the first incubation is detected at 420 nm. A second substrate incubation cycle then follows with, in this example, a reduced concentration of DTNB in the substrate. The second product peak is detected after a second stopped flow incubation period. The top line shows the pH trace detected by the on-line pH meter. To regenerate the column it is washed with acidic elution buffer. During this step in the method the detector wavelength is returned to 280 nm. The small peak in the elution segment reflects desorption of bound materials and the valve change.

Fig. 5 shows an overlay of the chromatograms for four samples, focusing only on the region of the second substrate peak from Fig. 4 at about 13.65 min. The 1 mm I.D. cartridge was used in these runs. There is some absorbance associated with a blank sample, owing to absorption of DTNB. The off-scale peak is generated from an amniotic fluid sample from a fetus undergoing demise. Degeneration of fetal tissue releases a large amount of many proteins, including AChE. The other two peaks are from samples judged to be normal (negative) or abnormal (positive) by the gel electrophoresis assay. In a limited set of ten samples, all of the positive samples gave readings higher than the negative samples. Clinically normal samples do have some AChE activity, as has been reported previously [12]. Inclusion of 50 $\mu M$ inhibitor BW284C51 with the substrate was sufficient to abolish all activity in both normal and abnormal samples. Based on experience to date, the 1 mm are superior to the 2.1 mm I.D. cartridges, in that they give a stronger positive signal and a lower blank signal

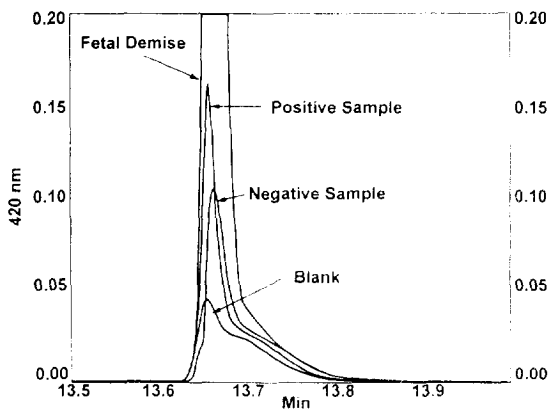


Fig. 5. AChE enzyme product. This figure shows an overlay of the chromatograms for four samples, focusing only on the region of the second substrate peak at about 13.65 min. In this case the 1 mm I.D. cartridge was used. There is some peak associated with a blank sample, owing to absorption of DTNB. The off-scale peak is generated from an amniotic fluid sample from a fetus undergoing demise. In this case the degeneration of the tissue releases a large amount of many proteins, including AChE. The other two peaks are from samples judged to be normal (negative) or abnormal (positive) by the gel electrophoresis assay.

and are less consumptive of reagents, particularly antibody.

Fig. 6 shows the analytical standards used to set up the SEC part of the experiment. Panels A and B show complete 2D assay chromatograms; depending on the step in the assay, the UV detector is either monitoring the effluent from the SEC column or the ID cartridge. In Fig. 6 the standard 2.1 mm I.D. cartridge and 2-ml reagent loop were used. The chromatogram segments are numbered to correspond to the steps outlined under Experimental and illustrated in Fig. 2. In Fig. 6A, a combination of hemoglobin and IgG was used to establish the molecular mass performance of the SEC column and select the timing of diverting a G4 AChE fraction to the cartridge. In this trace the first segment shows the SEC column alone in-line, and the next segment shows collection of a fraction from the SEC column on the ID cartridge. The third segment is from a wash of the ID cartridge and the peak reflects removal of unbound material from the ID cartridge. The ID cartridge is then taken off-line and the SEC continues, showing the elution of the IgG peak and the Hb peak. In this case the fraction collected on the ID cartridge just precedes the IgG peak, i.e., $M_r > 160\,000$. The substrate addition to the ID cartridge results in an off-scale peak in segment 5. This example is for a standard I.D. (100-$\mu$l) cartridge, where substrate is being added from an overfilled 2-ml loop, hence the large peak of substrate. The cartridge is taken off-line for the stopped flow incubation of 4 min and the SEC column is washed and regenerated in step 6. Following the wavelength change to 420 nm, the ID cartridge is inserted in-line and the product peak flows by the detector (step 7). The product peak in this case is only that associated with the DTNB blank.

Fig. 6B shows the results obtained for the purified G4 AChE from electric eel. There are minimal proteins detected at 280 nm during SEC during any step except step 6 (very low-molecular-mass materials). The AChE trapped on the ID cartridge gives a large product peak in step 7. This chromatogram shows that the timing of step 2, the collection on the ID cartridge, was correct for collecting G4 AChE.

Fig. 7 shows a chromatogram for an amniotic fluid sample. The same sequence of events as described in Fig. 6 occurs here. Some protein begins to emerge from the SEC column during the collection period on the ID cartridge, and is washed from the cartridge (steps 2 and 3). The majority of additional 280-nm absorbing material elutes near 5 min, after the end of the ID cartridge collection, in step 4. Some small materials continue to elute from the SEC column during the stopped flow incubation (step 6). The peak in step 7 is the product peak following the wavelength change, and reflects a positive sample.

Comparable results were obtained for both the AE-1 and 4F19 monoclonal antibodies. In the absence of the SEC column, i.e. the short single-column assay, both antibodies showed significant reactivity with G2 AChE. RBC-derived G2 AChE was negative in the two-column assay, as it should be, as it is of the wrong molecular mass to be collected during step 2 (data not shown).

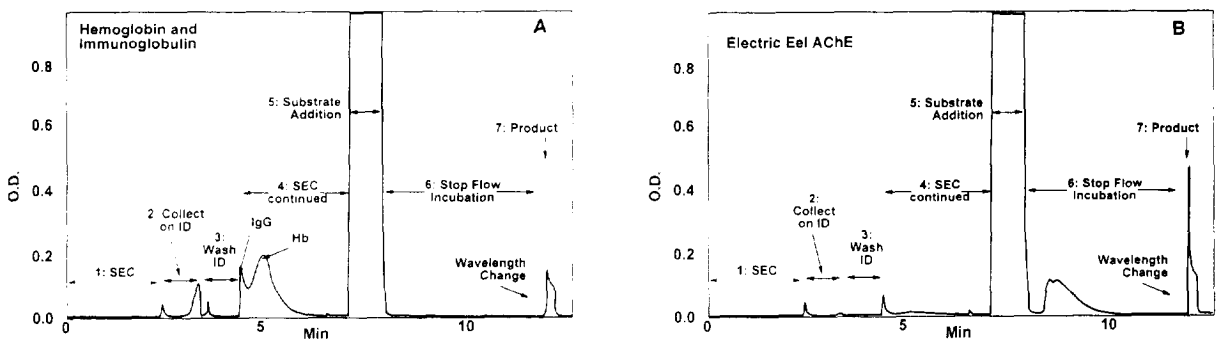


Fig. 6. Chromatograms for analytical standards showing the complete 2D assay. The first 13 min are monitored at 280 nm and reflect events largely controlled taking place on the SEC column. At 13 min the wavelength of the detector changes to 420 nm monitoring the product from the ID cartridge. The first set of standards used (A) was a combination of hemoglobin and IgG to establish the molecular mass performance of the SEC column and time the fraction collected on the ID cartridge. The segments of the chromatogram are numbered to correspond to the events in the method depicted in Fig. 2 and described under Experimental. In this trace the first segment has the SEC column alone in-line, the next segment collects a fraction off the SEC column on to the ID cartridge, the third segment washes the ID cartridge and the peak reflects removal of unbound material from the ID cartridge. The ID is then taken off-line and the SEC continues, showing the elution of the IgG and Hb peaks. In this case the fraction collected on the ID cartridge just precedes the IgG peak, i.e., $M_r > 160\,000$. The substrate addition to the ID cartridge results in our off-scale peak. This example is for a standard ID cartridge, were substrate is being added from an overfilled 2-ml loop, hence the large peak of substrate. The cartridge is taken off-line for the stopped-flow incubation of 4 min and the SEC column is washed and regenerated. Following the wavelength change to 420 nm the ID cartridge is inserted in-line, and the product peak flows by the detector. In the Hb and IgG sample, there is only the blank product peak. In (B), electric eel AChE was used as a positive standard for G4 AChE. In the sample there are mimimal proteins detected at 280 nm during SEC, except for very small materials, but there is a large product peak. This shows that the timing of the collection on the ID cartridge in step 2 is correct for capturing G4 AChE on the column.

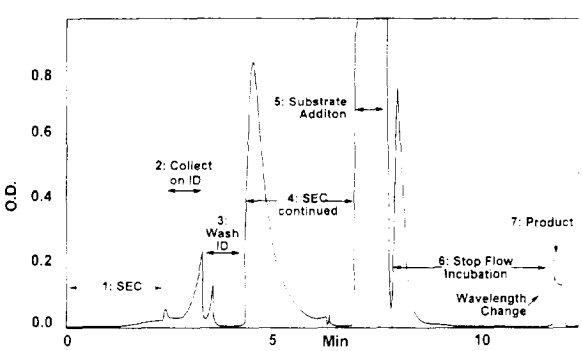


Fig. 7. Chromatogram for amniotic fluid sample. The same sequence of events as described in Fig. 6 occurs here. Some protein begins to emerge from the SEC column during the collection period on the cartridge ID, and is washed from the latter. The majority of additional 280 nm-absorbing material elutes near 5 min, after the end of the ID cartridge collection. Some continue to elute from the SEC column after the substrate addition step. The final peak is the product peak following the wavelength change.

## 4. Discussion

This paper reports the method development phase of a project to improve and automate the determination of AChE in amniotic fluid. A more extensive clinical evaluation is needed before any conclusions can be drawn about the clinical utility of this approach. However, we have established several important features of the assay. First, anti-AChE antibodies can be immobilized on ID cartridges and used to capture AChE from a flowing stream. Second, quantification of the enzyme, with sensitivity at clinically relevant levels, is possible using the on-column conversion of substrate into product. Third, we have illustrated multiple substrate addition steps, allowing the comparison of substrates and the testing of enzyme inhibitors. Fourth, the use of hybrid analytical methods, combining chromatographic separations with immunoaffinity capture, has been illustrated.

These chromatograms offer a wealth of in-

formation about the sample. For example, the SEC portion of the chromatogram gives a view of the total protein content of the sample, fractionated by size. During the enzyme detection portion of the chromatogram (steps 7 and later), one could measure the total captured AChE enzymatic activity with one substrate, activity in the presence of an inhibitor with a second substrate and total bound protein in the elution step. This would allow quantification of the fraction of active bound enzyme and the fraction of bound activity that is inhibited.

The implications of this study go beyond the determination of AChE in amniotic fluid. The assays illustrated here show the potential for chromatographic-based instrument systems to replace electrophoretic-based gels. Implicit in this replacement is the ability to access the computer-controlled automation, precision and range of sample sizes possible with modern HPLC instruments, none of which are available with gels. A recent review cites over 300 assays reported in the literature where *in situ* detection of enzyme activity in electrophoretic gels is used [13].

This assay also illustrates the linkage of molecular mass and antigen recognition. Further, currently Western blots are the only other assay format that allows linkage of antigen size information with immune recognition. Western blots suffer many of the same limitations of sample size, being semi-quantitative, time consuming and labor intensive, as do gel AChE assays. Replacement of Western blot technology with an HPLC-based system that provides equivalent information would be significant.

These experiments also illustrate the use of a second column to improve the selectivity of an immunoassay. The two immobilized antibodies evaluated did not discriminate adequately between G2 and G4 AChE until they were used in conjunction with the SEC column. In general in immunodiagnostics there can be a long search for a monoclonal antibody with ideal selectivity. Alternatively, the dual-column method illustrated allows the development of a protocol where antibody selectivity is not the only basis for selectivity in the assay, and an ideally specific monoclonal antibody is not needed. In the particular application of NTD diagnosis, the G2 form of AChE is so tightly bound to the erythrocyte membrane that it is probably not a clinically relevant factor. However, in cell and molecular biology studies of AChE, when multiple forms of AChE have been solubilized then this two-column assay allows the various forms to be determined independently.

The experiments illustrate the potential analytical power of dual-column assays, merging chromatographic separations with immunodetection. In the current context they offer the promise of a rapid, automated assay for AChE in amniotic fluid. This assay would allow the use of larger sample sizes than the currently used gels. Development of a completely validated clinical assay is in progress. As one example of a more general approach, however, this paper has implications for improving the specificity of immunoassays, for automating Western blot-like assays, and for measuring the specific activity of enzymes.

## References

[1] D. Zevin-Sonkin, A. Avni, R. Zisling, R. Koch and H. Soreq, J. Physiol. (Paris), 80 (1985) 221.
[2] A. Chatonnet and O. Lockridge, Biochem. J., 260 (1989) 625.
[3] N. Wald, H. Cuckle and K. Nanchahal, Prenatal Diagnosis, 9 (1989) 813.
[4] R.D. Barlow, H.S. Cuckle, N.J. Wald and C.H. Rodeck, Br. J. Obstet. Gynaecol., 89 (1982) 821.
[5] A.D. Smith, N.J. Wald, H.S. Cuckle, G.M. Stirrat, M. Bobrow and H. Lagercrantz, Lancet, (1979) 685.
[6] D.J.H. Brock and P. Bader, Clin. Chim. Acta, 127 (1983) 419.
[7] Y. Sadovsky, M.L. Robbin, B.F. Crandall, R.A. Filly and M.S. Golbus, Prenatal Diagnosis, 13 (1993) 1071.
[8] A.G. Rasmussen, K. Sorensen, J. Selmer, J. Zeuthen, O.J. Bjerrum, U. Brodbeck and B. Norgaard-Pedersen, Clin. Chim. Acta, 166 (1987) 17.
[9] D. Peat and D.J.H. Brock, Clin. Chim. Acta, 138 (1984) 319.
[10] N. Afeyan, N.F. Gordon and F.E. Regnier, Nature, 358 (1992) 603.
[11] J. Hangaard, K. Sorensen, U. Brodbeck and B. Norgaard-Pedersen, Scand. J. Clin. Lab. Invest., 44 (1984) 717.
[12] B.F. Crandall, W. Kasha and M. Matsumoto, Am. J. Med. Genet., 12 (1982) 361.
[13] G.P. Manchenko, Handbook of Detection of Enzymes on Electrophoretic Gels, CRC Press, Boca Raton, FL, 1994.