The relevance of buffer system ionic strength in immunoassay development

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Abstract

The best validated immunoassays for neurodegeneration have been developed for class III and IV intermediate filaments. There are a number of unique biochemical features of the intrinsically unstructured polyampholytic tail regions of these proteins which affect domain structure and thereby affinity and epitope recognition of antibodies used in immunoassays. Here one of these intermediate filaments, the neurofilament heavy chain, is chosen to demonstrate the effect of the ionic strength of a buffer system on the analytical signal to noise ratio. Higher ionic strengths gave better results. Next, a dose-dependent effect is demonstrated for barbitone to increase epitope recognition and protein quantification. The described effects of the buffer systems may be found helpful for future immunoassay developments.

Keywords biomarker, protein structure, immunoassay, polyampholyte, ionic strength, buffer, barbitone

Introduction

Intermediate filaments (IF) describe a class of proteins with assemble into polymers with a diameter of about 10 nm, which is intermediate between the larger myosin filaments ($\approx$ 15 nm) and the smaller actin filaments ($\approx$ 6 nm) [1, 2]. The last two decades in immunoassay development have been extremely successful in delivering some of the best validated and clinically useful IF biomarker assays to date [1, 3, 4].

Most IFs, such as the neurofilament heavy chain (NfH, are polyampholytes [5, 6]. From 607 residues of the NfH C–terminal tail region 310 (51%) are charged. Of these 156 are anionic and 154 cationic amino acid residues. The physical properties of polyampholytes in solution depend on key electrostatic properties of the buffer such as ionic strength, pH and ion concentration [7]. For neurofilaments (Nf) it was shown that modification of these properties had profound implications on Nf networks and gel formation [6, 7].

In vitro data on NfH using circular dichroism and atomic force microscopy strongly suggest the presence of about 70.8% of random coils with about 24–57% of unfoldable amino acids [8, 9]. Therefore NfH is not only a polyampholyte but also contains an intrinsically unstructured tail region [1, 10]. The 3D domain structure of the NfH tail region constantly changes under physiological conditions [6]. This has implications for immunoassays were NfH epitopes recognised by antibodies employed may be exposed or masked depending on the composition of the buffer system. In addition to these structural changes at the epitope level affecting antibody binding affinity, there are also pH related effects on affinity. Generally speaking a lower pH reduces antibody binding affinity such that lowering the pH can be used to select high affinity antibodies [11]. Inversely, high pH buffer systems can have advantages in increasing the binding of lower affinity antibodies.

This study tested the effect of different buffer compositions on quantification of NfH in a well established immuno-assay [1, 12].

Materials and methods

Antibodies

The capture or primary antibody was mouse monoclonal anti–NfH antibody (SMI clone 35) purchased from Covance Research Products (SMI-35R, Berkeley CA, USA). This IgG1 antibody binds with high affinity to a whole range of degree of phosphorylation of NfH [13, 14]. In the original papers the antibody was labelled as clone "03-44". The detection or secondary antibody was rabbit polyclonal anti-NfH purchased from Sigma (Sigma, N 4142, Lot: 091K4832) The indicator or tertiary antibody was horseradish peroxidase (HRP) labelled swine polyclonal anti–rabbit antibody and was purchased from DAKO (DAKO, Copenhagen, Denmark). The stock concentration of the antibodies can vary from lot to lot. It is important to remember that SMI35 is of very high affinity and will require sufficient dilution as excess of high affinity antibodies inhibits immunoreactivity. For this reason antibody dilutions used here were 1/5,000 for SMI35 and 1/1,000 for all other antibodies.

Chemicals

TRIS borate-EDTA buffer concentrate (catalogue number 93290-1L), Tris Base, sodium barbitone (C8H11N2NaO3, molecular weight 206.17 g/mol), barbitone (C8H12N2O3, molecular weight 184.19 g/mol), NaCl, ethylene–diamine–tetra–acetic disodium salt (EDTA), $NaHCO_3$, Na_2CO_3, Tween 20 and bovine serum albumin (BSA) were of analytical grade (Sigma). TMB was purchased from DAKO. Hydrochloric acid (HCl) was obtained from Merck (Darmstadt, Germany). NUNC Maxisorb Microtitre plates were obtained from Life Technologies (Paisley, Scotland).

Samples

Purified bovine NfH: Bovine HPLC purified NfH was obtained from Affiniti Research Products (UK).

Native human NfH: Fifteen CSF samples (10 mL each sample, collected from patients who underwent extra ventricular drainage for management of acute hydrocephalus) were collected in polypropylene tubes

and immediately centrifuged (5000 rpm for 5 minutes). Supernatants were aliquotted and stored at -80°C in 10 mL polypropylene tubes until analysis. In agreement with the Ethic Committee and the United Kingdom Human Tissue Act, all patient details were anonymised.

Buffer systems

Buffer A: barbitone buffer. Per litre H_2O (grade I) add 13.1 g sodium barbitone (63.5 mM), 2.1 g barbitone (11.4 mM), 0.25 g disodium EDTA (6 mM) and 0.2% BSA. Calibrate to pH 8.9 with HCl. *Buffer B:* TRIS borate-EDTA buffer concentrate 1:10 diluted in H_2O (grade I). Calibrate to pH 8.9 with HCl. *Buffer C:* TRIS buffered saline (TBS) buffer. Per litre H_2O (grade I) add 1.2 g Tris base (10 mM); 8.766 g NaCl (150 mM). Calibrate to pH 7.5 with HCl. *Wash solution:* add 0.2% BSA and 0.05% Tween20 to buffer A/B/C. *Block solution:* add 2% BSA to buffer A/B/C. *Sample diluent:* add 0.2% BSA to buffer A/B/C. Different molar concentrations of barbitone, Na–barbitone, carbonate or bicarbonate were added per experiment.

Analytical procedures

The microtitre plates were coated overnight with 100 μl of capture antibody diluted 1/5000 in 0.05 M carbonate buffer, pH 9.5 as described [12]. For the following steps of the procedure either buffer A, B or C were used throughout. The plate was washed with washing solution (1x). The plate was blocked with 150 μl of block solution. After washing (2x), 50 μl of sample diluent were added to each well. Fifty μl of HPLC purified bovine NfH or human CSF were then added in duplicate to the plate. The plate was incubated at room temperature (RT) for 1 h. After washing (3x), 100 μl of the second antibody diluted 1/1000 in sample diluent were added to each well and the plate was incubated for 1 h at RT. The microtitre plate was washed (3x) and HRP–labelled swine anti–rabbit antibody, diluted 1/1000 in sample diluent, was added and incubated for 1 h at RT. After a final wash (x5), 100 μl of TMB substrate were added. The plate was incubated for 20-25 min at RT in the dark, the reaction was stopped by adding 50 μl 1 M HCl and the absorbance (optical density, OD) was read at 450 nm with 750 nm as the reference wavelength.

Statistics

All statistical analyses and graphs were prepared using SAS software (version 9.4, SAS Institute, Inc., Cary, North Carolina, USA). Normality was tested graphically and using Shapiro–Wilk statistics. Comparison of more than two groups was performed with general linear models. Statistical significance was accepted for $p<0.05$.

Results

Buffer system

The signal to noise ratio differed significantly between the three buffer systems ($F_{44,133}$=122, $p< 0.0001$, Figure 1). This was mainly due to an increase of signal, rather then reduction of background. The barbitone buffer had a significantly better signal:noise ratio compared to the TBS–buffer for all 15 samples and 87% (13/15) of the samples analysed in TRIS–borate buffer (see '***', '**' and '*' in Figure 1). For native NfH the TRIS–borate buffer had a significantly better signal:noise ratio for 78% (7/9) of the samples, but not for purified NfH (see '+' in Figure 1).

Sample diluent compounds

Compared to neat CSF adding barbitone but not Na–barbitone, carbonate or bicarbonate significantly increased the OD ($F_{16,36}$=728, $p< 0.0001$, Figure 2). For barbitone there was a clear dose–response. For Na–barbitone the signal remained essentially unchanged. In contrast, addition of carbonate or bicarbonate dropped the signal to the level of noise.

TRIS- borate buffer There was a clear barbitone dose–response curve with an improved signal to noise ratio for both purified bovine NfH ($F_{5,12}$=13.12, $p< 0.0001$) and native human NfH from CSF ($F_{5,30}$=12.47, $p< 0.000$, Figure 3 A). The effect of barbitone on the signal to noise ratio was more marked for native NfH quantified from CSF compared to purified bovine NfH. In absolute values the signal (OD) increased from 0.3 to 1.6.

Barbitone buffer For purified bovine NfH there was no dose–response curve and for addition of 1 M barbitone the signal to noise ratio dropped ($F_{5,12}$=256, p$<$ 0.0001 for all comparisons, , Figure 3B). For native human NfH (CSF) presence of barbitone (0.03 to 0.5 M barbitone) improved the signal to noise ratio 4- to 5–fold. There was a trend for a dose–response curve, but for addition of 1 M barbitone the signal to noise ratio dropped to 1 ($F_{5,30}$=7.6, p$<$ 0.0001 for all comparisons). The effect of barbitone on the signal to noise ratio was more marked for native NfH quantified from CSF compared to purified bovine NfH.

Discussion

This methodological study describes a strong, dose depended effect of a high ionic strength buffer on the quantification of an important native type III IF, NfH. Knowledge of the relationship between buffer ionic strength and NfH quantification will be informative for future immunoassay development in the IF biomarker field [1, 2].

First, comparison of three buffer systems demonstrated an over 5–fold better signal to noise ratio for quantifying NfH^{SMI35} for the barbitone buffer compared to either TBS–buffer or TRIS–borate buffer. For human CSF samples the TRIS–borate buffer also showed a better signal to noise ratio compared to the TBS buffer, but much less so than the barbitone buffer. This is consistent with the NfH^{SMI35} being a polyampholyte [5, 7] which forms tighter networks possibly masking some epitopes the higher molar salt concentrations [6], 150 mM NaCl in the TBS buffer compared to the TRIS–borate and barbitone buffers containing EDTA. The markedly better signal to noise ratio of the barbitone buffer compared to the TRIS–borate buffer further suggests that presence of barbitone rather then only pH or ionic strength [7] is important for unmasking the eloquent epitope/epitopes.

Therefore the effect of barbitone and its water soluble salt on quantification of NfH^{SMI35} were investigated in comparison to the physiological bicarbonate buffer system. This experiment showed a clear dose–response between the molar concentration of barbitone and amount of NfH^{SMI35} quantified from human CSF. There was no change of signal after adding the water–soluble Na–barbitone. Whether or not this points towards interactions of the more lipophilic barbitone with hydrophobic regions such as the α–helical body region [6] is difficult to say from the

present data. This would need to be addressed by use of different antibody binding pairs, ideally directed to different NfH phospho-epitopes in detailed affinity studies [11].

The marked difference between the effect of barbitone on purified bovine and native human NfH suggests that probably not, because the structure of the α–helical body region is very stable and highly preserved between mammals [15]. An alternative explanation may be that there are post–translational modifications of NfH which are relevant to antibody binding affinities and remain intact in human CSF, but get modified following the *in vitro* protein purification method used for the bovine NFH investigated here [16]. Repeating the experiments with more gentle protein purification methods allowing to study the neurofilament networks *in vitro* may clarify this question [6]. There is earlier experimental evidence that a barbitone concentration of 0.5–2 mM influenced the ultra structure and polymerisation of axonal intermediate filaments [17]. Barbitone also arrests rapid axonal transport [18] and delays dendritic growth [19].

Conclusion

Taken together, the present and previous [1, 17–19] data suggest an effect of a high ionic strength buffer system on the structure of NfH which exposes/masks epitopes and likely alters affinity relevant for quantification in immunoassays.

Conflict of interest

AP reports no conflict of interest.

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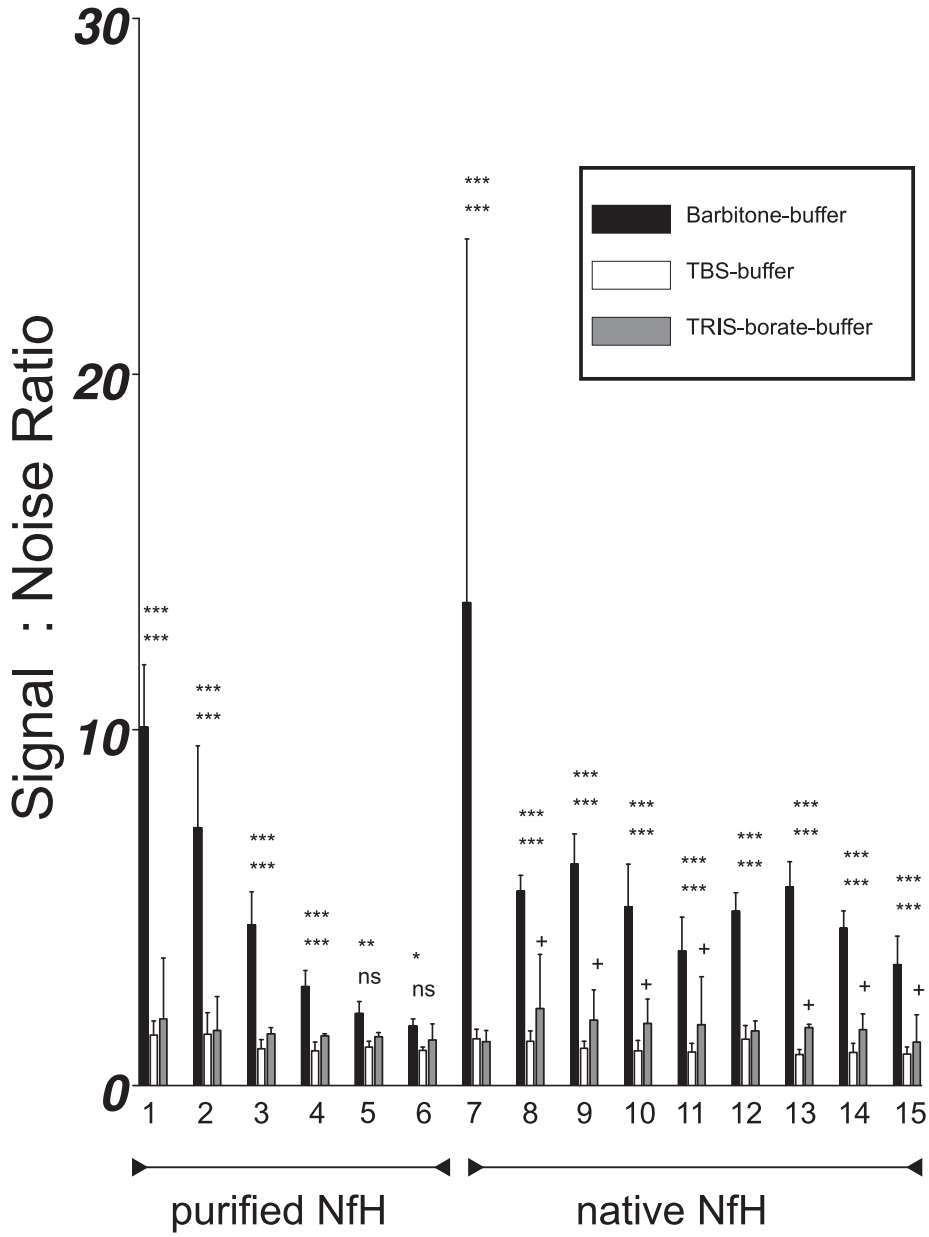

Figure 1: *A significantly better signal:noise ratio for NfH^{SMI35} is achieved with the barbitone buffer compared to either TBS or TRIS-borate buffers ($F_{44,133}$=122, p< 0.0001). The level of significance is indicated for comparing barbitone with TBS buffer in the top row and barbitone with TRIS–borate in the second row as *** = p<0.0001, ** = p<0.001, * = p<0.01, ns = not significant; TBS buffer with TRIS–borate as + = p<0.05.*

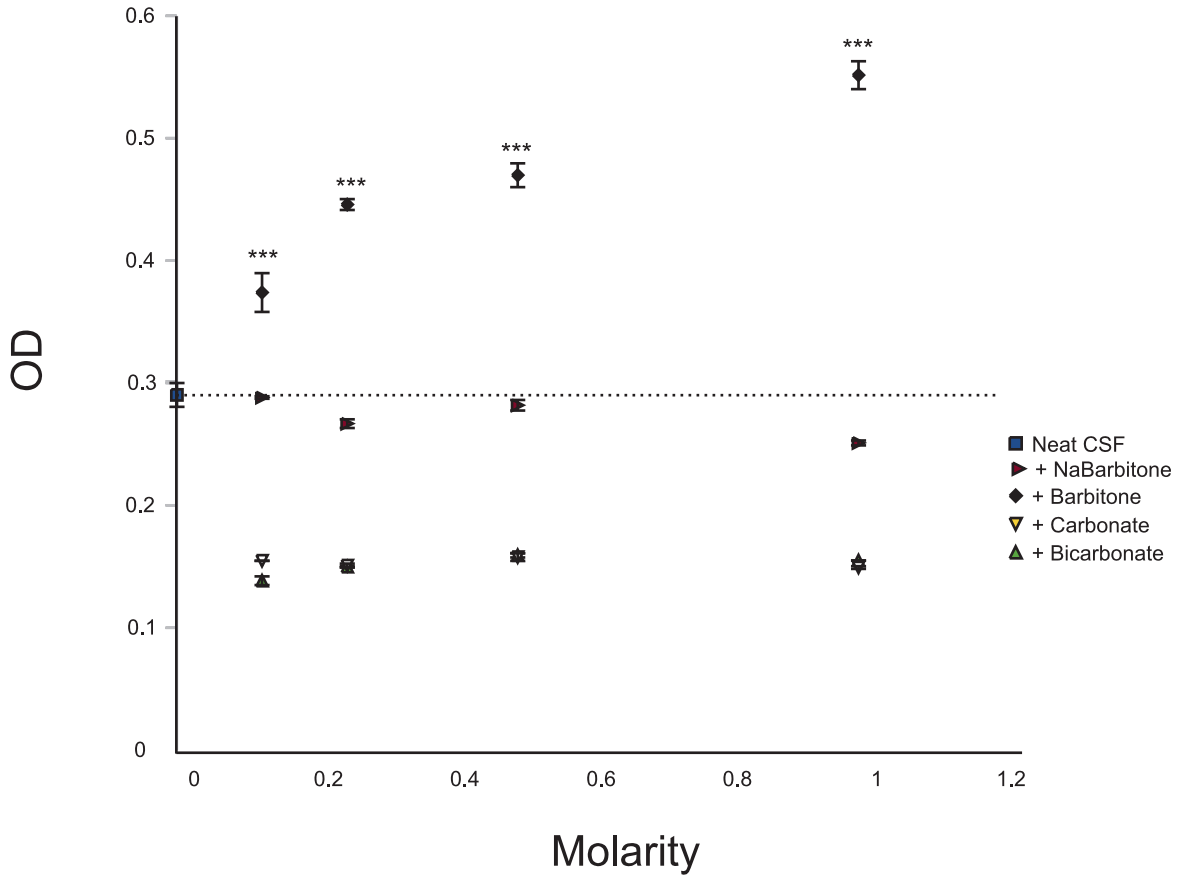

Figure 2: *Dose relationship between molarity of barbitone added to a CSF sample (black dot) and gain of signal compared to the neat CSF sample (grey square, horizontal reference line). There was no gain of signal for adding Na-barbitone (right–sided triangle) and loss of signal to the level of noise for adding carbonate (upside–down triangle) or bicarbonate (triangle). The mean±standard deviation are shown. *** = $p<0.0001$, OD = optical density (450 nm test, 750 nm blank).*

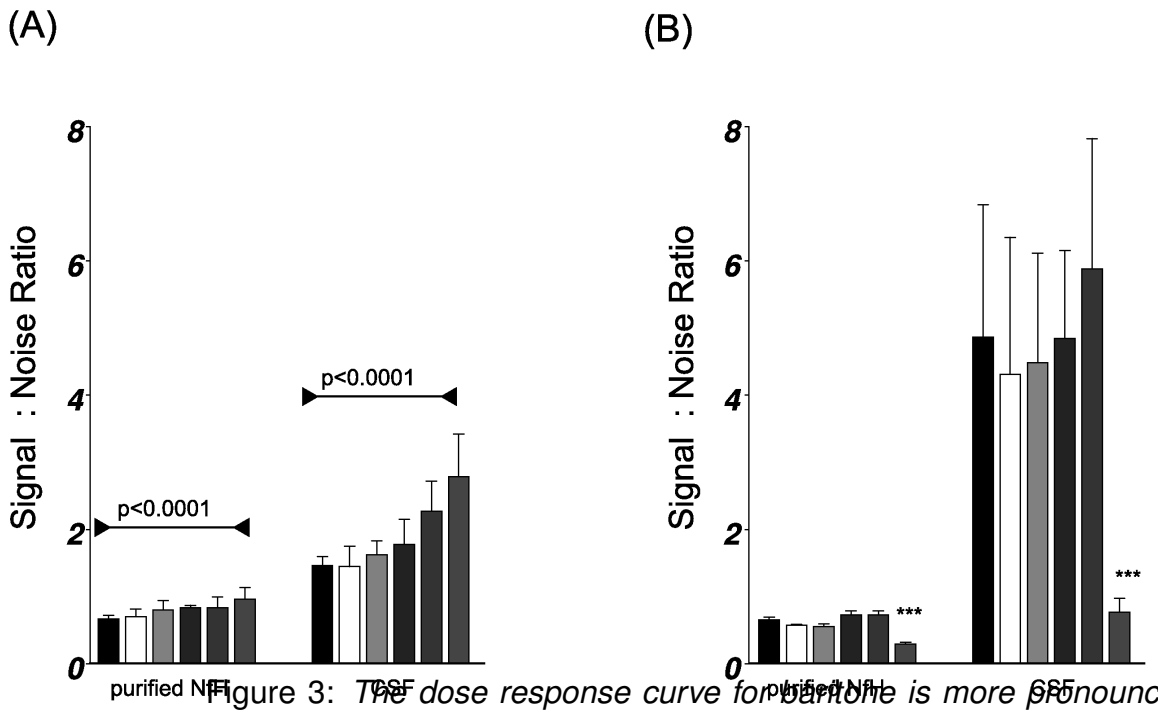

Figure 3: *The dose response curve for baritone is more pronounced for native human NfH quantified from the CSF compared to purified bovine NfH from either (A) TRIS–borate buffer or (B) Na–barbitone buffer.*