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Reversed-phase HPLC with UV detection for the determination of *N*-acetylaspartate and creatine

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In an earlier study, using hippocampal slice cultures from rat, we showed that *N*-methyl-D-aspartate (NMDA)¹ receptor activation caused release of *N*-acetylaspartate (NAA), an organic anion of predominantly neuronal origin that is frequently used as a marker of neuronal death and/or dysfunction [1,2]. A specific number of other organic anions, including the antioxidant GSH, were also released after NMDA receptor activation. The efflux of these anions was Ca²⁺ dependent and correlated with delayed nerve cell death measured 24 h later, indicating a role of the efflux in the nerve cell death. Interestingly, the efflux of NAA, as determined by reversed-phase HPLC coupled to UV detection, was accompanied by the release of a few unknown UV-absorbing components. The tight correlation between the efflux and delayed nerve cell death prompted us to try to identify the unknown components. In this work, we identified one of the earlier unknown and released substances as creatine (Cr) and further developed the previously used reversed-phase HPLC method [1].

The HPLC system consisted of a Varian 5000 pump, a Waters 717 autosampler, and an Applied Biosystems 757 UV detector. For multiple wavelength measurements, a Waters 2996 photodiode array detector was used. The sampling rate was 1 spectrum/s, and the sampling range was 190–400 nm with a resolution of 1.2 nm. Data were processed with Millennium³² software (Waters, Milford, MA, USA). If not indicated, chemicals were from Sigma (St. Louis, MO, USA). NAA (Aldrich, Milwaukee, WI, USA) and Cr were separated at room temperature on a TSK gel ODS-80T_M column (250 × 4.6 mm, 5 μm particle size, Tosoh, Tokyo, Japan) fitted with a guard column packed with C₁₈ Nucleosil material. The mobile phase consisted of 50 mM NaH₂PO₄ (pH 2.15) and was degassed with N₂ before use. A flow rate of 1 ml/min was used, and peaks were detected by absorbance at 210 nm. After each sample set (typically 20–40 injections of 90 μl each), the column was washed with 20 and 100% methanol (Rathburn, Walkersburn, UK) for 40 min each.

For NAA analysis, the current method was compared with that of Koller and coworkers. This method was carried out essentially as described previously [3].

The detection limits at a signal-to-noise ratio of 2 were 9 pmol (0.1 μM) injected for NAA and 1.8 pmol (0.02 μM) injected for Cr. To determine the linearity and intraassay precision of the method, six sets of eight combined standards, ranging from 0.5 to 200 μM for NAA and from 0.1 to 40 μM for Cr, were injected. Interassay precision was determined by injecting six sets of the combined standards (stored at –20 °C) over a period of 32 days. Calibration curves were plotted as peak height (μV) versus analyte concentration (μM). At a signal-to-noise ratio of 10, the method was linear from 45 pmol up to at least 18 nmol injected (0.5–200 μM) for NAA

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¹Abbreviations used: NMDA, *N*-methyl-D-aspartate; NAA, *N*-acetylaspartate; Cr, creatine; CV, coefficient of variance; ACSF, artificial cerebrospinal fluid.

and from 9 pmol up to at least 3.6 nmol injected (0.1–40 μ M) for Cr. The inter- and intraassay coefficients of variance (CVs) for NAA and Cr were generally less than or slightly greater than 10%, and calibration curves showed R^2 values greater than 0.999, demonstrating good precision and linearity. The accuracy was determined by adding a known amount of NAA and Cr to samples prior to injection. The recoveries for NAA and Cr were 101.6% (7.6% CV, $n = 6$) and 99.5% (6.2% CV, $n = 6$), respectively. A chromatogram of a mixture of NAA, Cr, and possible interfering compounds is shown in Fig. 1A. As can be seen, a large number of organic ions were separated. NAA and Cr eluted with retention factors (k) of 3.4 and 1.3, respectively ($k = (t_R - t_0)/t_0$, where t_R is the retention time of the analyte and t_0 is the elution time for unretained solutes). The lack of an absorbance maximum at 217 nm confirmed that the Cr peak was free of creatinine (Fluka, Buchs, Switzerland) (Fig. 1B) [4]. Furthermore, for NAA analysis, the current method was compared with that of Koller and coworkers [3]. No significant difference was found between the concentrations obtained with the two methods when brain tissue extracts were analyzed ($P = 0.67$, $n = 29$, paired t test).

By lowering the pH in the mobile phase to 2.15, organic acids such as NAA and Cr are protonated and retained on a reversed-phase column. Although the use of pure aqueous buffer in combination with silica-based columns is generally not recommended, we found that our method was robust in terms of peak shape and column lifetime. We chose NAA and Cr detection at 210 nm, although both compounds show higher absorbance at lower wavelengths. However, at lower wavelengths, the increasing absorbance of the void interfered with Cr analysis. The separation of Cr and creatinine could be confirmed by the characteristic absorbance of creatinine at 217 nm in low-pH solution (Fig. 1B) [4]. In addition, no difference between concentrations obtained with our method and that of Koller and coworkers was found when NAA was analyzed. This further confirms the validation of both methods. The method is comparable in sensitivity to earlier methods using UV detection for NAA analysis and is better when compared with some recently published reversed-phase methods for Cr determination [3,5–7]. Previous methods for NAA analysis recommended purification before injection or use of ion-pairing reagents and extensive washing between injections [3,7]. Also, it was reported that a high K^+ concentration interfered with NAA determination using the method of Koller and coworkers [8]. With our method, a high K^+ concentration (100 mM) did not interfere with NAA or Cr analysis (data not shown), and we showed previously that high salt-containing samples, such as artificial cerebrospinal fluid (ACSF), can be analyzed with the current method [1]. Thus, in some applications, our method may be a suitable complement, particularly to the method based on anion exchange, which is the most commonly used HPLC method for NAA determination today [3]. We have also determined NAA and Cr in extracts from rat hippocampus developed in vivo and in vitro (Fig. 2 and Table 1). The increasing concentrations of NAA and Cr during in vivo development are in agreement with previous studies; however, as can be noted, the NAA concentration, but not the Cr concentration, is lower in hippocampus developed in vitro than in vivo [3,9,10]. Reversed-phase HPLC methods for the separate determination of NAA and Cr have been reported previously, but to our knowledge the current method is the first one that evaluates potential interferences with NAA analysis and allows the simultaneous determination of NAA and Cr [5,6,11,12]. In addition, a large number of other metabolites, including *N*-acetylaspartylglutamate, were separated, making the method a possible choice in a variety of different types of neuro-chemical studies.

In conclusion, the method described is a relatively simple reversed-phase HPLC method for the simultaneous determination of NAA and Cr. The method shows good precision and linearity and can be used for NAA and Cr determination in ACSF as well as in brain extracts and, thus, is a good complement to existing methods for NAA and Cr determination.

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Fig. 1.

(A) Chromatogram of a standard of NAA, Cr, and possible interfering compounds (nmol injected in parentheses): 1, hypotaurine (0.5); 2, aspartate (45); 3, NMDA (90); 4, glutamate (45); 5, *L*-carnosine (0.9); 6, γ -aminobutyric acid (90); 7, Cr (11); 8, pyruvate (18); 9, *N*-acetylhistidine (1.3); 10, ascorbate (4.5); 11, lactate (99); 12, ATP (1.3); 13, ADP (0.45); 14, α -ketoglutarate (18); 15, NAA (14); 16, GSH (2.7); 17, maleate (0.6); 18, citrate (45); 19, succinate (9); 20, AMP (0.6); 21, *N*-acetylglutamate (14); 22, fumarate (0.6); 23, urate (4.5); 24, *N*-acetylaspartylglutamate (2.7); 25, adenosine (2.3); 26, inosine (2.7). Taurine was undetectable at 9 nmol injected. (B) Separation of the Cr and creatinine (Crn) peaks (left) and their different profiles of absorption (right).

Fig. 2.
(A) Chromatogram of UV-absorbing species at 210 nm in an extract of a rat hippocampus. (B) and (C) show the absorbance profiles of the NAA and Cr peaks, respectively, in a standard injection and in an extract of a cultured hippocampal slice from rat.

Content (nmol/mg protein $\pm$ SEM) of NAA and Cr in hippocampus (in vivo, $n = 6$) and in cultured hippocampal slices from rat (in vitro, $n = 12-14$)

Table 1

Age of tissue (days)	NAA in vivo	NAA in vitro	Cr in vivo	Cr in vitro
8-9		—		—
15-16	19.5 ($\pm$ 1.2) *#	27.9 ($\pm$ 2.0) *	62.2 ($\pm$ 3.8) *	81.3 ($\pm$ 6.5)
19-22	55.8 ($\pm$ 1.6) *#	24.7 ($\pm$ 1.0)	104.1 ($\pm$ 2.5) *	84.1 ($\pm$ 7.7)
28-30	64.7 ($\pm$ 2.5) *#	25.7 ($\pm$ 1.2)	104.0 ($\pm$ 4.0) *	95.8 ($\pm$ 7.2) *
	66.0 ($\pm$ 2.5) *#		108.3 ($\pm$ 4.7) *	

Note. Hippocampal slices for culturing were obtained from 8- to 9-day-old rats.

* $P < 0.05$ compared with postnatal days 8-9.

# $P < 0.05$ compared with age-matched cultured slice. Analysis of variance with Tukey's post hoc test.