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Journal of Chromatography B, 830 (2006) 196–200
JOURNAL OF CHROMATOGRAPHY B
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A novel, quantitative assay for homocarnosine in cerebrospinal fluid using stable-isotope dilution liquid chromatography–tandem mass spectrometry

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Received 19 August 2005; accepted 26 October 2005
Available online 22 November 2005

Abstract

We describe a rapid and sensitive method for the quantification of homocarnosine in physiological fluids, with particular emphasis on cerebrospinal fluid (CSF). Homocarnosine was quantified as the butyl derivative, with $^2H_2$-L-homocarnosine as internal standard. Following deproteinization of CSF samples, supernatants were evaporated to dryness and derivatized with 10% 6 M HCl in butanol. Samples were chromatographed on a C_{18} column and detected by liquid chromatography–tandem mass spectrometry (LC–MS/MS) operating in the multiple reaction monitoring mode. The intra- and inter-assay variations were 4.6 and 10.9%, respectively. Mean recovery of homocarnosine at two concentrations was 105%. The limit of detection in CSF approximated 20 nmol/L. CSF homocarnosine is age dependent and ranges from <0.02 to 10 μmol/L. Our method is applicable to the analysis of CSF derived from patients with heritable defects in the GABA pathway, patients with homocarnosinosis or serum carnosinase deficiency, and should be applicable to other model systems in order to further explore the biological role and significance of homocarnosine in mammalian systems.

Keywords: Homocarnosine; GABA; Tandem mass spectrometry; Succinic semialdehyde dehydrogenase deficiency

1. Introduction

Homocarnosine is a brain specific dipeptide of γ-aminobutyric acid (GABA) and L-histidine [1] in the mammalian central nervous system [2] (Fig. 1). Large quantities of GABA and GABA-conjugates, including homocarnosine, are found in the CSF derived from patients with GABA-transaminase deficiency. These patients are unable to transaminate GABA to succinic-semialdehyde, resulting in increased CSF GABA [2].

CSF homocarnosine may also be elevated in patients with heritable succinic-semialdehyde-dehydrogenase deficiency (SSADH-deficiency), in which accumulated succinic-semialdehyde is blocked from entry into the Krebs-cycle and is subsequently reduced to γ-hydroxybutyric acid (GHB), the biochemical hallmark of SSADH deficiency [2]. GABA is hypothesized to accumulate in SSADH deficiency secondarily to overall GABA pathway dysfunction. The only remaining human genetic defect in which homocarnosine accumulates is a single isolated family with homocarnosinosis, in which the mother and several children accumulated supraphysiologic levels of CSF homocarnosine [2]. The accumulation of CSF homocarnosine was linked with serum carnosinase deficiency in this isolated family, indicating that carnosinase deficiency and homocarnosinosis in this family are apparently one disorder, despite the fact that CSF carnosine was not increased [2]. Increased CSF homocarnosine has not been demonstrated in other patients with isolated serum carnosinase deficiency [2]. The exact biologic function of homocarnosine in mammalian systems remains almost completely unknown. Specific brain regions manifest homocarnosine concentrations >1 mM, and in selected neuronal tracts homocarnosine may serve as a

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doi:10.1016/j.jchromb.2005.10.053

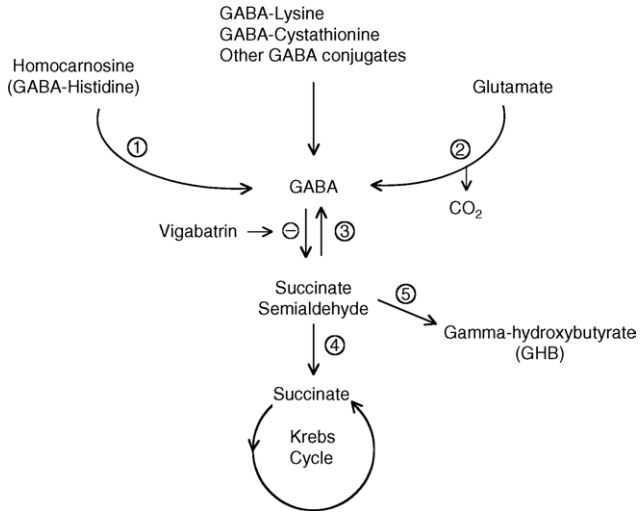

Fig. 1. Interrelationships of homocarnosine and GABA metabolism. Numbered enzymes include: 1, carnosinase; 2, glutamic acid decarboxylase; 3, GABA-transaminase (pharmacological site of action of vigabatrin); 4, succinate semialdehyde dehydrogenase (site of the heritable metabolic block in humans and mice with γ-hydroxybutyric aciduria); and 5, succinate semialdehyde reductase. Not all steps in this pathway are shown. Abbreviation: GABA, γ-aminobutyric acid.

repository for GABA production [3]. There is expanding evidence that homocarnosine provides neuroprotection relative to excitatory and free-radical damage mechanisms, at least in vitro [4–6]. Moreover, there is emerging data suggesting that homocarnosine in the central nervous system manifests antiepileptic properties [7]. To further examine the biological roles of homocarnosine, it will be fundamentally important to develop systems in which its levels are significantly perturbed, while simultaneously having available quantitative methods for detecting these perturbations.

To date, the available methods for quantifying homocarnosine include high performance liquid chromatography (HPLC) with fluorescence detection [8,9] or low-capacity cation-exchange HPLC with UV-detection [10]. Neither method employs a labeled internal standard for quantification. Another disadvantage of these existing methods is the large single chromatographic run time of 55 min with a retention time of homocarnosine of 20–30 min.

In the current report, we describe a high-throughput and sensitive isotope-dilution method for quantifying homocarnosine in physiological fluids, with particular focus on CSF derived from control individuals and those with defects in the GABA degradative pathway. Extensive HPLC separation is not required by the use of the high selectivity of the MS/MS. The use of the labeled internal standard corrects for sample-to-sample differences in extraction performances. The relatively short time needed for the sample pre-treatment and the short analysis time (single chromatographic run time of 4 min) makes this method suitable for large-scale measurements. Our method is completely applicable to other physiological fluids and tissue extracts, and represents a new tool with which to further explore the biological importance and role of the brain-specific GABA-histidine dipeptide homocarnosine.

2. Materials and methods

2.1. Materials

γ-Aminobutyryl-L-histidine (L-homocarnosine) was obtained from Sigma (Zwijndrecht, Netherlands). $^2H_2$-L-Homocarnosine was synthesized using histidine and $^2H_2$-γ-aminobutyric acid ($^2H_2$-GABA), which was purchased from Nippon Sanso (Tokyo, Japan), and the product was confirmed by proton NMR. The NMR spectra of unlabeled and labeled homocarnosine are identical with only one change. In the NMR spectrum of unlabeled homocarnosine, the methylenegroup from the GABA-backbone next to the keto-carrying carbon, shows as a triplet at 2.4 ppm. In case of $^2H_2$-L-homocarnosine the triplet signal at 2.4 ppm is absent due to the presence of $^2H_2$ at this carbon. Isotopic purity of $^2H_2$-L-homocarnosine was found to be 93%.

Ethanol (min. 99%) and butanol were purchased from Merck (Darmstadt, Germany). HPLC-grade methanol was obtained from VWR (Amsterdam, The Netherlands) and ammonium acetate was purchased from Sigma.

2.2. Sample preparation

To 100 μL CSF aliquots 10 μL of 0.1 mM $^2H_2$-L-homocarnosine in water was added.

The samples were deproteinized by adding 300 μL ethanol (99%). The mixtures were mixed thoroughly and centrifuged at 20,000 × *g* for 5 min at 4 °C. The clear supernatants were transferred to vials and evaporated to dryness under a stream of nitrogen at 40 °C. 100 μL of a 10% 6 M HCl in butanol solution was added and the samples were left to derivatize at 60 °C for 15 min. After cooling, the samples were evaporated to dryness at 40 °C under a stream of nitrogen. The residues were redissolved in 100 μL mobile phase which consisted of H_2O–methanol (50:50, by volume) containing 25 mM ammonium acetate. The solutions were transferred to new tubes and centrifuged for 5 min at 10,000 × *g* at 4 °C. One to ten microliters of the clear supernatants were injected onto the LC/MS/MS system.

Aqueous calibrators containing 0, 0.1, 0.5, 1.0 and 5.0 nmol homocarnosine were also processed as described above. The concentration of the CSF analyte was calculated by interpolation of the observed analyte/IS peak-area ratio into the linear regression line for the calibration curve, which was obtained by plotting the peak-areas ratios against analyte concentration.

2.3. LC/MS/MS-analysis

The HPLC system consisted of a Perkin-Elmer Series 200 HPLC pump, a Perkin-Elmer Series 200 autosampler, and a Harvard Apparatus Pump 11 infusion pump. Detection was performed on an API 3000 triple quadrupole tandem mass spectrometer (PE-Biosystems Sciex, Nieuwerkerk a/d IJssel, Netherlands). Chromatography was performed on a Symmetry C_{18} analytical column (3.9 mm × 150 mm; 5 μm bead size; Waters) using H_2O–methanol (50:50, by volume) containing 25 mM ammonium acetate as mobile phase. The flow was set

to 0.9 mL/min and was split into a ratio of 1:4 producing an inlet flow of 180 μL/min into the mass spectrometer. The turbo ion electrospray source was operating in the positive mode and the temperature was set at 400 °C; turbo ion gas (nitrogen) was employed at a flow rate of 8 L/min and the ion spray voltage was set at 4200 V; declustering potential was set at 30 V and the collision energy was 20 V. Data were acquired and processed using Analyst for Windows NT (version 1.3.1). For the multiple reaction monitoring (MRM) the following transitions were performed, *m/z* 297.0 to *m/z* 212.0 for homocarnosine and *m/z* 299.0 to *m/z* 212.0 for $^{2}H_{2}$-L-homocarnosine.

GABA measurements were performed by stable isotope dilution electron-capture negative-ion mass fragmentography, as previously described [11].

3. Results

3.1. Mass spectra

Product ion scans of unlabeled and labeled L-homocarnosine were derived to obtain the specific fragmentation pattern of these compounds. In the MS/MS spectra of both labeled and unlabeled L-homocarnosine a fragment of *m/z* 212.0 was detected as the major ion. This loss of fragments 87 and 85 for labeled and unlabeled L-homocarnosine, respectively, is due to the release of the aminobutyryl part of the molecules within the collision cell.

By measuring the transition of protonated L-homocarnosine, *m/z* 297.0 and $^{2}H_{2}$-L-homocarnosine, *m/z* 299.0 (quadrupole 1) to fragment *m/z* 212.0 (quadrupole 3) multiple reaction monitoring (MRM) experiments were performed.

3.2. Chromatography

A representative MRM chromatogram of L-homocarnosine in CSF is depicted in Fig. 2. This figure also shows the chromatogram of a CSF sample derived from a patient with SSADH deficiency, with elevated L-homocarnosine.

Table 1
Accuracy data for the LC–MS/MS method

	Mean ± S.D. (μmol/L)	CV (%)	Recovery (%)
Homocarnosine added, μmol/L ($n = 5$)			
0	2.17 ± 0.10		
2.0	4.32 ± 0.08	1.9	108
5.0	7.31 ± 0.35	4.8	103

3.3. Linearity, precision, recovery and limit of detection

The calibration curve was linear with concentrations of unlabeled homocarnosine up to 50.0 μmol/L. Validation experiments were performed with pooled CSF samples. The intra-assay variation was found to be 4.6% (2.17 ± 0.10 μmol/L, $n = 5$). The inter-assay variation was 10.9% (2.29 ± 0.25 μmol/L, $n = 9$). Two different homocarnosine concentrations were employed for recovery experiments. The mean recovery for both concentrations was 105% with coefficient of variations (CV) of less than 5%. The data are tabulated in Table 1.

To estimate the limit of detection in CSF samples, we verified the peak height of homocarnosine and the noise in the chromatographic region of homocarnosine. Using this approach, the minimal detectable concentration, at a signal to noise ratio of >5, was approximately 20 nmol/L.

3.4. Reference values for homocarnosine in human CSF

Control values for homocarnosine in human CSF were obtained by analysis of samples ($n = 73$) from patients whose CSF GABA concentrations were previously shown to be within our control range, and who were not suspected to suffer from a metabolic disorder. The age range varied from 2 months to 81 years. No significant gender difference was observed. However, the homocarnosine concentration was highly age-dependent, as illustrated in Fig. 3.

Fig. 4 depicts the differences in CSF homocarnosine values between two control groups (individuals <10 years and those >10 years of age) as compared to CSF samples obtained from SSADH-patients in the age-range of 2 months to 10 years.

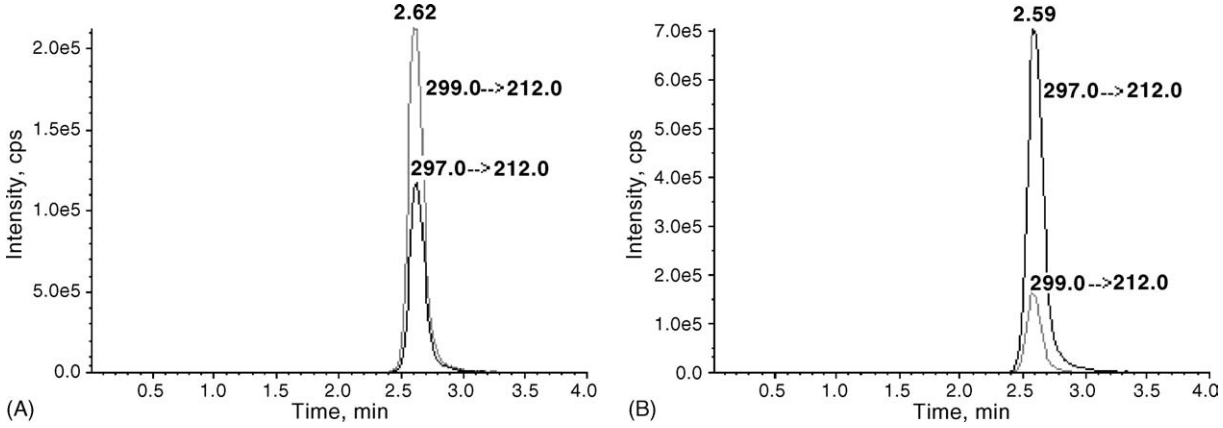

Fig. 2. Chromatograms of pooled CSF sample (A) and SSADH-deficient patient (B) (age of 4 years).

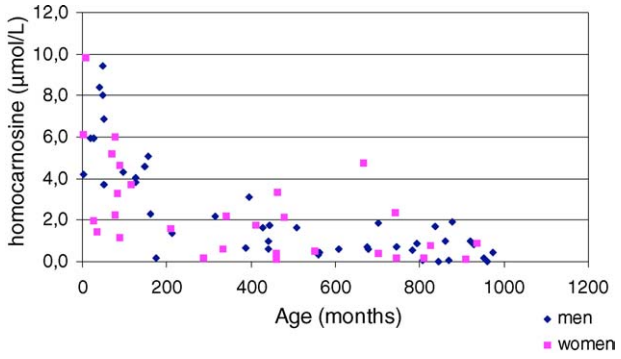

Fig. 3. Control concentrations of Homocarnosine in CSF stratified by gender.

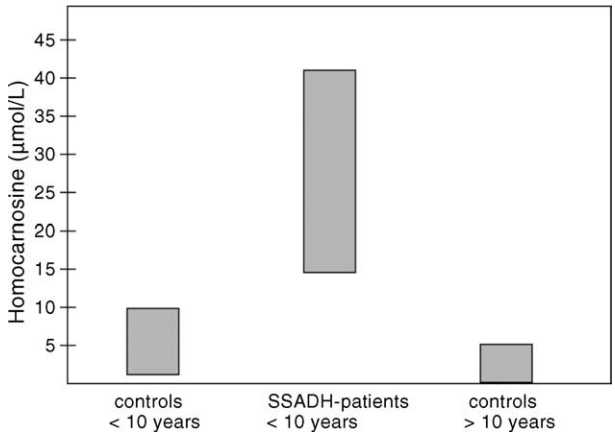

Fig. 4. CSF homocarnosine concentrations (ranges) in controls and patients (mean values for controls <10 years = 5.1 μmol/L (n = 21) (1.12–5.84 μmol/L); controls >10 years = 1.3 μmol/L (n = 52) (<0.02–5.06 μmol/L); SSADH-deficient patients <10 years (n = 9) = 25.7 μmol/L (14.7–41.1 μmol/L)).

To characterize the potential relationship between GABA and homocarnosine in CSF, we also determined the total GABA levels in all control samples using gas chromatography mass spectrometry (GC/MS) [11]. Total GABA in CSF derived from control individuals was 1.3 to 10.8 μmol/L. As for homocarnosine, total GABA decreases with age and there was no significant gender difference.

As shown in Fig. 5, there was a highly significant linear correlation between total GABA and homocarnosine in control CSF.

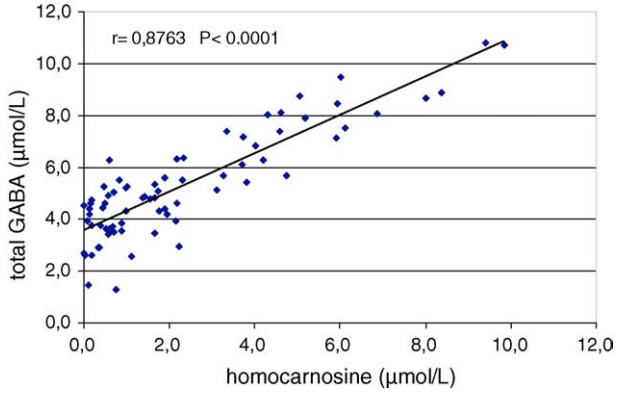

Fig. 5. Linear correlation for total GABA and homocarnosine in CSF in samples derived from control individuals (n = 73; age 2 months to 81 years).

4. Discussion

Validation data for the current method indicates that our new assay is sensitive, reproducible and accurate. Advantages of this method are the relatively short sample clean-up time and rapid analysis. For control individuals, homocarnosine concentrations varied between <0.02 to 10 μmol/L and were age-dependent, with the CSF homocarnosine level decreasing with age. This finding is in direct contrast with the earlier literature, in which it was shown that homocarnosine actually was higher in adults than neonates [12]. Only in two controls (age 70 and 80 years) we found a homocarnosine concentration lower than 0.02 μmol/L, the detection limit of our method. In the controls with age <10 years we did not find a homocarnosine concentration lower than 1.12 μmol/L. An in vivo homocarnosine concentration less than 0.02 μmol/L will only be expected in persons at higher age.

Since GABA can derive from homocarnosine hydrolysis, we also quantified total GABA in all the control samples using GC/MS, in order to investigate whether there was a close relationship between the two metabolites. The data of Fig. 5 reveals a highly significant linear correlation, and it is noteworthy that in all control samples the total GABA concentration (derived through hydrolysis of CSF samples) was higher than the homocarnosine concentration. Clearly, a significant level of GABA-conjugates other than homocarnosine exists in human CSF, most likely comprised of GABA-lysine, GABA-cystathionine, and other minor components [2]; conversely, in contrast to GABA and homocarnosine, histidine (the other component of homocarnosine that is not a neurotransmitter) concentrations are not age dependent [13].

The assay described should be applicable to other physiological fluids in addition to CSF; as well, it will be an important adjuvant with which to study model systems in which homocarnosine metabolism is hypothesized to be disrupted, both naturally and using artificial (pharmacological) interventions. A number of groups have employed the irreversible GABA-transaminase inhibitor vigabatrin to block normal GABA metabolism and assess the physiological alterations [14,15], and in vivo NMR studies have documented that vigabatrin intervention elevates brain homocarnosine as well as GABA [15]. In animal systems undergoing vigabatrin intervention, our assay will enable quantitation of homocarnosine in discrete brain regions following drug administration.

Animal models in which GABA metabolism is disrupted are much rarer, however. One of these is SSADH deficiency, and in the corresponding murine knockout model of this disorder, we have previously documented an increase in total and regional concentrations of homocarnosine in brain extracts using our newly developed assay [16]. There is no other known mammalian knockout model in the pathway that we are aware of at this time (Fig. 1). However, deletion of SSADH in plants results in accumulation of GHB and stress-related accumulation of reactive oxygen intermediates [17]; it would be of interest to examine these plant systems to ascertain if homocarnosine is also increased, and it should be feasible to disrupt other enzymes of the GABA metabolic pathway in the plant system.

We conclude that our rapid, sensitive and highly specific assay for homocarnosine should be a valuable tool with which to study both normal and pathophysiological systems in which GABA metabolism, and ultimately homocarnosine homeostasis, is disrupted. These studies should serve to provide a more comprehensive understanding of the normal physiological function of homocarnosine in mammalian and non-mammalian systems.

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