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Measurement of CGRP in dried blood spots using a modified sandwich enzyme immunoassay

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Abstract

Calcitonin gene-related peptide (CGRP) has roles as a neurotransmitter, neuromodulator and trophic factor. CGRP has been reported to be elevated in neonatal blood of children with autism or mental retardation as compared with normal subjects by recycling immuno-affinity chromatography (RIC). While CGRP detection in neonatal blood is thus important, it is not easy to detect CGRP in dried blood spots because of the limitations of sample volume and the specificity and the sensitivity of available assay systems. In the present study, we modified a "Sandwich Enzyme Immunoassay" for the purpose of detecting CGRP in blood spot eluate. We have prepared blood spots from blood collected from normal human subjects and measured CGRP level in eluates from these blood spots. Instead of a purification step, we have introduced a pre-incubation step and used washed erythrocytes as a dilution solution. The modified assay has good recovery and specificity and appropriate dilution curves. We have compared the eluate levels with levels in serum and plasma from the same individuals and find that CGRP levels in blood spot eluate were similar to those of serum and plasma. Thus, the newly modified EIA may be a useful method for the detection of CGRP in blood spot eluate.

Keywords: EIA; CGRP; Dried blood spot

1. Introduction

Autism is a complex brain disorder that causes a range of developmental problems. A wide variety of neural abnormalities in autism have been described, but a biologic diagnostic test has not yet been developed (Lord et al., 2000). Recently, for the neuropeptides vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), changes in neurodevelopmental disorders such as autism, mental retardation, Down syndrome and cerebral palsy have been reported (Nelson et al., 2001). Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide occurring in one of two forms both in human (CGRP-I and CGRP-II) and in the rat (CGRP-α and CGRP-β). CGRP-I results from alternative splicing of the calc I gene that also encodes for calcitonin whereas CGRP-II is the only known product of the calc II gene (Nelkin et al., 1984; Steenbergh et al., 1986; Poyner, 1992; Rossum et al., 1997; Frobert et al., 1999). CGRP is widely distributed in the brain and cardiovascular system, thyroid gland and gut. Main sources of CGRP are neurons of the central nervous system, specifically a subpopulation of primary sensory neurons. CGRP serves as a transmitter/neuromodulator in neurotransmission, a neuromediator in the cardiovascular system (Rossum et al., 1997) and also a powerful endogenous vasodilator (Brain et al., 1985). CGRP levels are increased in patients with thyroid carcinoma (Mason et al., 1986; Carter et al., 1991; Schifter and Johnsen, 1994) or pheochromocytoma (Mouri et al., 1989) and, as noted, in neonatal blood from infants with neurodevelopmental disorders (Nelson et al., 2001). Conventionally, CGRP concentration has been measured by RIA (Schifter, 1991; Wimalawansa, 1993), EIA (Seth et al., 1988; Frobert et al., 1999) and RIC (Nelson et al., 2001). Due to low blood levels of CGRP and the small size of some of the samples such as blood spots

Abbreviations: Ab, antibody; AchE, acetylcholine esterase; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; LOD, limit of detection; LOQ, limit of quantitation; PACAP, pituitary adenylate cyclase activating peptide; PBS, phosphate-buffered saline; PHI, peptide histidine isoleucine; RIA, radio immunoassay; RIC, recycling immuno-affinity chromatography; S.D., standard deviation; VIP, vasoactive intestinal peptide

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that are available, a sensitive and reliable assay that allows the precise quantitation of this peptide in different biologic media is needed for the study of CGRP function and distribution in disease.

In the present study, we evaluated a commercial EIA kit with blood specimens from normal human volunteer donors. From these specimens, we have prepared three types of samples: serum, plasma and eluates from dried blood spots. We have modified a commercial enzyme immunoassay kit to detect CGRP successfully in these blood samples.

2. Materials and methods

2.1. Reagents and instrumentation

The CGRP EIA kits and CGRP affinity columns were obtained from Spi-Bio Inc. (Massy Cedex, France). Protease Inhibitor cocktail was purchased from Calbiochem (San Diego, CA). ELISA microplate spectrophotometer (Molecular Devices, Menlo Park, CA), SOFTmax^{TM} software (Molecular Devices), C18 Sep columns (Millipore, Bedford, MA), spectrophotometer (ThermoSpectronic, Woburn, MA). Secretin, pituitary adenylate cyclase activating peptide (PACAP), peptide histidine isoleucine (PHI), vasoactive intestinal peptide (VIP) and substance P was purchased from BACHEM (Califonia, CA). Somatostatin was purchased from Sigma (St. Louis, MO).

2.2. Collection of samples

Healthy adult human blood samples (serum, plasma and whole blood) were collected from the NIH Blood Bank after obtaining informed consent from the subjects. Samples were collected into each of the following blood collection tubes—(a) plasma: EDTA (ethylenediaminetetra-acetic acid); (Vacuette®, Greiner Labortechnik, Austria); (b) serum: SST-serum separator (Vacutainer®, Becton Dickinson Vacutainer System USA, Rutherford, NJ, USA); (c) whole blood: non-treated tube (Vacutainer®). Blood samples containing EDTA or separator were centrifuged to separate plasma and serum from whole blood.

Whole blood samples were spotted onto filter paper and dried for approximately 3 h over an open non-absorbent surface at room temperature. The dried blood spots were stored at −20 °C until use. Before the analyses were performed, five 5 mm diameter circles were punched from the dried blood spot on the filter paper for each subject and were eluted in 250 μl of phosphate-buffered saline (PBS) overnight at 4 °C. We have found that the volume of blood per 5 mm disk was ∼10 μl, so a five-fold dilution of the blood occurred during the elution. Eluate was filtered using microspin columns with 10 μm frits (Amersham Biosciences, Piscataway, NJ) centrifuged at 15,000 rpm for 20 min.

2.3. Protein measurement

Total protein was measured for each subject at 500-fold dilutions using a spectrophotometer (ThermoSpectronic) to determine absorbance at 260 and 280 nm.

2.4. Preparation of CGRP free media

Normal patient plasma and serum were applied to a column to which CGRP antibody had been absorbed; the plasma or serum were incubated overnight on the column at 4 °C and then eluated. For the washed erythrocyte solution, whole blood was collected in 5 ml of EDTA vacutainer tubes and centrifuged at 3000 rpm for 15 min. Plasma and the buffy coat were removed and discarded. About 3 ml of normal saline was added and mixed for 5 min and then centrifuged. The saline and the buffy coat were removed. This washing step was repeated three times.

2.5. Determination of CGRP by EIA

The assay principle is that of a sandwich enzyme immunoassay. CGRP in standards and five-fold diluted samples were added to wells on a plate which had been coated with anti-CGRP antibody. CGRP that is bound to this primary antibody in turn binds a secondary that has been conjugated with acetylcholine esterase (AchE). The amount of secondary antibody is revealed when it is reacted with Ellman's reagent.

To avoid strong matrix effects and the necessity for an extraction and purification procedure, we used CGRP free plasma/serum or washed erythrocytes as a dilution solution. CGRP standard solutions and QC solutions were reconstituted and diluted with CGRP free plasma, serum or washed erythrocytes instead of the EIA buffer. CGRP in serum, plasma and eluate was measured using the CGRP EIA kit (Spi-Bio Inc.) according to the manufacturer's protocol. A critical change was that CGRP in eluate was pre-incubated with the antibody-coated plate overnight at 4 °C before being reacted with the tracer antibody (the AchE-conjugated antibody). That is, 100 μl of standards and samples were added to individual wells in a 96-well microtiter plate and incubated at 4 °C for overnight. Two hundred microliter of tracer antibody was then added and incubated overnight at 4 °C. Ellman's reagent was then added to the wells and incubated 30 min to 1 h at RT with shaking. A standard curve was generated for each assay and the concentration of CGRP in each sample was determined using the standard curve. The values were expressed as the mean of duplicate determinations. Plates were read at 450 nm with a microplate spectrophotometer (Molecular Devices) and results were analyzed with SOFTmax^{TM} software (Molecular Devices).

3. Results

3.1. Detection limit and detection range

The sensitivity of immunoassays is expressed as either the limit of detection (LOD) or limit of quantitation (LOQ). The former is the lowest concentration that can be confidently interpreted as being different from zero; this corresponds to a signal 3S.D. above the mean for the blank solutions. The LOQ is the lowest concentration that gives a signal interpretable as representing a particular concentration and is set at 6S.D. above blank.

In our study, the LOD for CGRP in serum and plasma was <3.9 pg/ml and the LOQ was 7.8 pg/ml. When eluate samples are