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Identification and determination of succinyladenosine in human cerebrospinal fluid

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Abstract

Succinyladenosine (S-Ado) is a biochemical marker of adenylosuccinase deficiency – the genetic defect of purine de novo synthesis. S-Ado has been previously reported as normally undetectable in cerebrospinal fluid (CSF) of children not suffering from this defect. In present study, we employed solid-phase extraction and thin-layer chromatography for isolation of a compound with spectral and chromatographic characteristics identical to S-Ado from human CSF. The high-performance liquid chromatography–negative-ion electrospray ionization mass spectrometry analysis confirmed that the isolated compound is S-Ado. We established the reference values of S-Ado in CSF of children (1.1 ± 0.4 μmol/l; mean±S.D; $n=26$) by means of reversed-phase HPLC method on a C_{18} column with UV detection.

Keywords: Succinyladenosine

1. Introduction

The purine nucleoside succinyladenosine (Fig. 1), *N*-(9-β-D-ribofuranosyl-9H purin-6-yl)aspartic acid (S-Ado), is the metabolic product of dephosphorylation of intracellular adenylosuccinic acid (S-AMP) by cytosolic 5-nucleotidase. S-AMP is the intermediate in the purine nucleotide interconversion between inosine monophosphate (IMP) and adenosine monophosphate (AMP), catalysed by enzymes adenylosuccinate synthase (EC 6.3.4.4.) and adenylosuccinate lyase (ASL, EC 4.3.2.2.).

The presence of high concentrations of S-Ado in the human cerebrospinal fluid (CSF) in the range of 100–500 μmol/l is a diagnostic marker for inherited enzyme deficiency of ASL. Neurological involvement is the principal symptom in most patients suffering from ASL deficiency. Thin-layer chromatography (TLC) [1,2], high-performance liquid chromatography (HPLC) [3,4] and capillary electrophoresis (CE) [5] methods for determination of S-Ado in the diagnosis of this genetic defect have been published previously. However, the concentrations of S-Ado are reported to be normally undetectable in CSF of control subjects (i.e., under the detection limit of the system used) [3,5,6,7]. Only Abelskov

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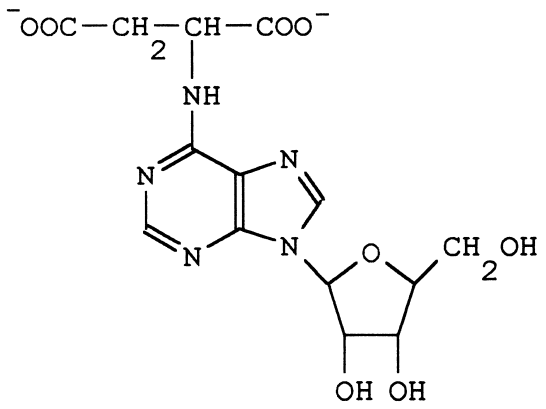

Fig. 1. Chemical structure of succinyladenosine.

has reported, in 1959, in a preliminary note the presence of S-Ado in human CSF [8].

We have reported previously the finding of a peak with identical retention and UV characteristics as S-Ado in the CSF of subjects not suffering from ASL deficiency [9]. The present study describes the isolation and identification of the S-Ado in human CSF by means of solid phase extraction (SPE), TLC and HPLC–mass spectrometry (MS). The reference values and ranges of S-Ado concentrations in the control CSF of children were determined using a reversed-phase HPLC method.

2. Experimental

2.1. Reagents

All solvents were of analytical or HPLC-grade and obtained from Merck (E.Merck, Darmstadt, Germany). Adenylosuccinic acid, ammonium acetate and potassium phosphate, alkaline phosphatase (E.C.3.1.3.1) were obtained from Sigma (Sigma-Aldrich, Prague, Czech Republic).

2.2. Standards

The S-Ado standard was prepared from adenylosuccinic acid by treatment with alkaline phosphatase according to Ref. [10]. Concentration of the S-Ado standard was determined by UV spectrophotometry (UV–Vis Spectrometer Lambda 10, Perkin-Elmer, Beaconsfield, UK) at 268 nm. The molar extinction coefficient of corresponding nucleotide ($19.2 \cdot 10^3$ l $mol^{-1}cm^{-1}$) [11] was used for the calculation.

2.3. Control samples

The CSF used in present study was obtained from subjects who were undergoing a diagnostic lumbar puncture for the investigation of suspected inherited metabolic disorder. All subjects had negative ASL deficiency urinary screening test performed according to Ref. [12]. The age of the subjects ranged from 4 weeks to 7 years. The CSF samples were stored at −20°C until analysis. The samples were analysed within two weeks after collection, no significant changes in S-Ado concentrations were detectable during this period.

The S-Ado was isolated from 5 ml of CSF pooled from the rests of the samples after laboratory tests for metabolic disorders.

2.4. Isolation of S-Ado from CSF

2.4.1. Solid-phase extraction procedure

The disposable SPE cartridge filled with 500 mg of C_{18} sorbent (Separon TM SGX C_{18}, 60 μm, Tessek, Prague, Czech Republic) was conditioned by successive aspiration of 5 ml methanol, 5 ml of water and 5 ml of 50 m*M* phosphate buffer, pH 2.5.

A 5-ml aliquot of CSF acidified to pH 2.5 with 2 *M* H_3PO_4 was loaded slowly onto the cartridge. Following the wash with 5 ml of 50 m*M* phosphate buffer, pH 2.5 and 3 ml of water, the S-Ado was eluted with 2 ml of 20% methanol and the fraction (S-Ado fraction) was concentrated under the stream of nitrogen at 50°C to a volume of 200 μl. An aliquot of each fraction was taken for the HPLC analysis. The extraction recovery was calculated from the peak area of S-Ado in the S-Ado fraction and the area of S-Ado in the neat CSF sample before extraction.

2.4.2. TLC separation

A 100-μl volume of the concentrated S-Ado fraction was applied as a 5 cm line on a high-performance TLC (HPTLC) silica gel plate (Art. 5633, Silica gel 60, 10×10 cm; Merck). Approximately 1 nmol of S-Ado standard was applied on a

separate silica gel HPTLC plate. Both plates were developed in ethyl acetate–acetic acid–water (13:4:4).

The plate with S-Ado standard was sprayed with naphtoresorcinol/sulphuric acid reagent [13] and heated at 105°C for 5 min. The R_F of the blue reacting band of S-Ado standard was determined and the band with corresponding R_F was scraped off from the plate with CSF sample. The S-Ado was eluted from the silica with 2×200 μl of water and the fraction was concentrated by evaporation under a stream of nitrogen at 50°C.

The concentrated S-Ado containing CSF fraction (50 μl) and the S-Ado standard (approximately 1 nmol) were applied as two separate 4 cm lines on a Cellulose F aluminium sheet (Art. 5574, 10×10 cm, Merck) TLC plate and developed in butanol–acetic acid–water (4:1:1). The S-Ado band was detected by background fluorescence quenching under UV light at 254 nm. The band with the R_F corresponding to the band of S-Ado standard was scraped off and eluted with 400 μl of water. The eluate was concentrated under the stream of nitrogen at 50°C to a volume of approximately 100 μl.

2.5. HPLC–MS analysis

The S-Ado fraction isolated from CSF by SPE and TLC was analysed by a system consisting of a quaternary gradient Waters 616 HPLC pump directed by Waters 600S controller. A Rheodyne Model 7125 injection valve (Cotati, CA, USA) with a 5-μl sample loop was used for sample injection. A 5 μm Nucleosil ODS reversed-phase column (250×2.0 mm I.D., Macherey Nagel, Düren, Germany) was used for separation of S-Ado. Isocratic elution with 7 m*M* ammonium acetate–methanol (90:10, v/v) was performed at a 0.15 ml/min flow-rate at ambient temperature (23±2°C) Chromatographic column was linked either to the UV detector set at 268 nm or alternatively to the electrospray (ESI) interface of the mass spectrometer.

Negative-ion ESI mass spectra were recorded on the double-focusing instrument Finnigan MAT 95 (Finnigan MAT, Bremen, Germany) of BE geometry equipped with a Finnigan atmospheric pressure ionization (API) ion source. The S-Ado standard diluted with the mobile phase to concentration of 250 μmol/l was continuously infused through a stainless steel capillary held at 3.0 kV into the ion source via a linear syringe pump at a rate of 30 μl/min (Harvard Apparatus 22). A mixture of polypropylene glycols (average M_r=425, Aldrich, Steinheim, Germany) was used to calibrate the m/z scale of the mass spectrometer. Mass spectra were acquired in the 50–500 mass range, the scan duration was 5.0 s.

2.6. HPLC analysis of CSF

The HPLC system consisted of two Waters Model 501 pumps, (Millipore, Milford, MA, USA) controlled by an IBM personal computer with a Data Apex CSW software (DataApex, Prague, Czech Republic) Rheodyne Model 7125 manual injection valve and Waters Model 990+ UV photodiode array detector.

The CSF analyses were performed by modification of the method of Morris et al. [4]. A 50-μl volume of neat CSF were injected onto the SGX C_{18}, 250×4.0 mm I.D., 5 μm HPLC column (Tessek, Prague, Czech Republic). The linear gradient ran from 100% solvent A (3% methanol in 40 m*M* ammonium acetate, adjusted to pH 5.00 with glacial acetic acid) to 100% solvent B [methanol–acetonitrile–tetrahydrofuran–ammonium acetate (40 m*M*, pH 5.00) 26.1:3:3:67.9, v/v] over 30 min using a flow-rate of 0.8 ml/min. Prior to each subsequent analysis, the column was reconditioned with buffer A for 15 min. The analyses were performed at ambient temperature (25±1°C). All buffers were filtered and degassed by passage through a 0.45-μm filter under vacuum.

2.7. Validation

The linear relationship of peak areas to S-Ado concentrations was tested by replicate HPLC analysis of five standard solutions, containing concentrations of 0.1, 0.5, 1, 5 and 10 μmol/l. Each standard solution was injected in triplicate onto the column. The calibration graph generated by non-weighted linear regression was used for quantification of S-Ado in CSF.

Pooled CSF (control) sample was prepared to determine the reproducibility and accuracy of the method. The mean S-Ado concentration in the CSF control sample was determined by replicate (n=8)

HPLC analyses. Five consecutive analyses of CSF control sample (S-Ado=1.1 μmol/l) were performed to obtain the intra-day reproducibility data. The inter-day reproducibility was determined by replicate analyses of CSF control sample on five separate days within two weeks. The results obtained were expressed in terms of coefficient of variation (CV).

Intra- and inter-day accuracy was tested by analysis of CSF control sample spiked with known quantities of S-Ado standard. To 0.9 ml of the CSF control sample 100 μl of S-Ado standard at three different concentrations (0.2, 1, 5 μmol/l) was added. The resulted theoretical concentrations of S-Ado in the spiked samples were calculated. The accuracy was defined as the percent difference between the mean found and the theoretical concentration of S-Ado in the sample.

3. Results and discussion

3.1. Solid-phase extraction

The presence of succinyladenosine in all fractions was controlled by HPLC analysis. The majority of the compound was eluted in the S-Ado fraction. Suppression of ionization with a low pH phosphate buffer ensured sufficient retention of S-Ado on the C_{18} sorbent. The washing step with 50 m*M* phosphate buffer and water should remove more polar interfering compounds from the sample, while 20% methanol eluted S-Ado from the column, leaving the more nonpolar interferences retained on the sorbent. The recovery of S-Ado in the S-Ado fraction was better than 90%, traces of succinyladenosine were detected in the aqueous fraction.

3.2. TLC separation

We employed two TLC methods for the clean-up of S-Ado from the CSF sample. Both methods are modifications of screening methods routinely used in our laboratory. The identity of the R_F values of the compound isolated from CSF and the S-Ado standard on different TLC media should be a further indication of identity of both compounds. Furthermore, the presence of interfering compounds should be minimized in the sample for HPLC–MS analysis using the separation on both TLC systems. The R_F of the S-Ado standard on Cellulose plates determined under UV light (254 nm) was 0.49. The R_F of the blue reacting band of S-Ado standard after naphtoresorcinol staining on Silica plate was 0.55. The bands with R_F identical to S-Ado standard were scraped off from the TLC plates with CSF sample and eluted with water. The HPLC analysis of the eluates confirmed the presence of a compound in CSF with identical retention and UV characteristics as the S-Ado standard. The presence of UV absorbing interfering compounds in the CSF sample was minimized using the separation on the two different TLC systems.

3.3. HPLC–MS analysis

The measurement in the positive-ion mode did not provide any reasonable results. On the other hand, negative ion mass spectrum of the standard (Fig. 2) revealed the deprotonated molecule $[M-H]^-$ at m/z 382 as a base peak. Additional ions at m/z 425, 404 and 97 could be rationalised as $[MK-H]^-$, $[MNa-H]^-$ and phosphate, respectively. The low concentration of succinyladenosine in our real sample (100 μg/l) did not permit to perform the in-source collisionally-induced dissociation to obtain the fragmentation of molecular ion. After retuning the nozzle-skimmer potential difference both the

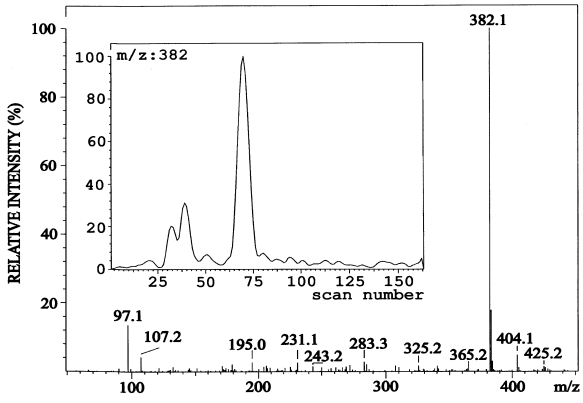

Fig. 2. Negative ion ESI mass spectrum of succinyladenosine standard. Inset: selected ion chromatogram of m/z 382 (deprotonated molecule) of real sample HPLC–MS analysis.

$[M-H]^-$ and required fragment ions disappeared in the chemical noise.

However, the identity of t_R and negative ion ESI mass spectra of S-Ado standard and the major peak detected in selected ion chromatogram of m/z 382 of the real sample obtained by isolation from CSF confirmed the presence of S-Ado in human CSF.

3.4. HPLC analysis

The HPLC analysis of neat CSF revealed a peak with identical retention time (15.2 min) and UV spectrum (maximum absorbance at 268 nm) as the S-Ado standard (Fig. 3). The separation between S-Ado peak and other metabolites presented in the human CSF depends on the pH of the mobile phase used. At lower pH (4.8) the S-Ado peak interferes with inosine, while at higher pH (5.2) S-Ado co-elutes with xanthine. At pH 5.00 the resolution was sufficient to allow quantification of S-Ado in the CSF sample. The concentrations of S-Ado detected in CSF of children (4 weeks to 7 years of age) were in the range of 0.2–2.4 μmol/l; 1.1±0.4; mean±SD, n=26).

3.5. Validation

The peak areas plotted versus S-Ado concentrations in the range 0.1–10 μmol/l resulted in a linear curve passing through the origin. The obtained regression equation $y=591.17x$; $r^2=0.9996$ (y and x represent area and concentration, respectively) was used for quantification of S-Ado in CSF. The differences between the nominal concentrations of S-Ado standards and concentrations calculated from the regression equation (less than 10%) indicate that the response is linear and the regression model is appropriate.

The S-Ado concentration in the pooled control sample used for method validation determined by HPLC was 1.1±0.04 μmol/l; (mean±SD; n=8). The CVs calculated for the intra- and inter-day precision were 4.7% and 1.7%, respectively. Three samples with nominal concentrations of S-Ado (1.2, 2 and 6 μmol/l) were prepared by addition of S-Ado standard to the CSF control sample. The percent differences between nominal (1.2, 2 and 6 μmol/l) and measured concentrations were 8.1, 5.1 and 2.2% for the samples analysed on the same day and were 8.9, 5.6 and 3.4% for the samples analysed on

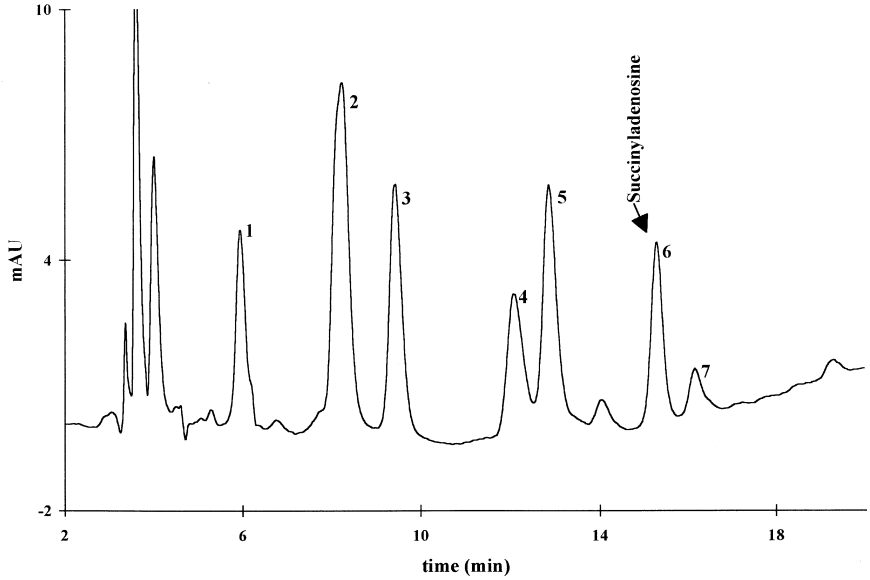

Fig. 3. Chromatogram obtained after injection of 50 μl of a human CSF sample on reversed-phase column. The concentration of S-Ado in the sample is 1.2 μmol/l. Chromatographic conditions as in Experimental. Peaks: 1=pseudouridine, 2=uric acid, 3=uridine, 4=hypoxanthine, 5=xanthine, 6=succinyladenosine, 7=inosine.

different days. The limit of detection (defined as signal-to-noise ratio of 3) calculated for S-Ado in CSF using the HPLC protocol reported here was 0.1 μmol/l.

4. Conclusions

Although the presence and significance of many nucleosides have been studied in human CSF [14–16], the values of S-Ado concentrations have been reported only in patients suffering from ASL deficiency [3,5–7], an inherited metabolic disorder of purine biosynthesis. This compound have been reported to be normally undetectable in control CSF in several publications. Using a HPLC method with UV detection we found S-Ado as a compound normally presented in the CSF of children (1.1 ± 0.4 μmol/l; mean±SD; $n=26$). To confirm the presence of S-Ado in human CSF, we have isolated the compound by means of SPE and TLC methods. The chromatographic properties of the isolated compound and S-Ado standard were identical under different chromatographic conditions. HPLC–MS analysis performed in the selected ion monitoring mode at m/z 382 confirmed the presence of S-Ado in human CSF.

The S-Ado concentrations in human CSF are above the detection limit of most recently used HPLC systems. Thus, the S-Ado peak may have been reported as an unknown compound in previous studies of purine nucleosides in CSF. The concentrations of other purine metabolites (hypoxanthine, xanthine, inosine, a.o.) are also in the low micromolar range in the human CSF. Depending on the pH of the mobile phase used, S-Ado can interfere either with xanthine, inosine or other metabolites on different C_{18} columns, causing a significantly incorrect quantification of these compounds.

Our result should allow better interpretation of HPLC analyses of purine nucleosides in CSF and should be a contribution to further studies of the role and metabolism of purines in the central nervous system.

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