Adenylosuccinate Lyase Deficiency- A cause of seizures in children
Development of an assay for its diagnosis

UCL-Institute of Neurology

MSc program in clinical Neurology

Adenylosuccinate Lyase Deficiency- A cause of seizures in children

Development of an assay for its diagnosis, a report submitted as partial fulfillment criteria for MSc.

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Dedication

To the soul and spirit of my father Elfatih Alsanousi,

To my mother Rabab Alsanousi,

To all children and families suffering from seizures and mental retardation.

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ABSTRACT

Background

Adenylosuccinate Lyase (ADSL) Deficiency is one of the rare causes of neonatal and infantile seizures. This condition results from autosomal recessive mutations in the gene coding for the enzyme ADSL, mapped to human chromosome 22q13.1-13.2. The ADSL enzyme plays an important role in the *de novo* pathways of purine metabolism and thus deficiency, and/or decreased catalytic activity of this enzyme is manifested in form of repeated seizures in early childhood. Other neurological symptoms and signs may be present, such as psychomotor retardation, autistic features, axial and peripheral hypotonia, and muscle atrophy and secondary feeding problems leading to severe growth retardation.

Although ADSL deficiency is considered a rare cause of childhood epilepsy and only 50 cases have been reported till now world wide, the magnitude of the problem can not be determined unless rapid accurate diagnostic test is introduced, especially, in communities with high rate of consanguinity. Fortunately, several reports have given a hint for Succinyladenosine or S-Ado as a new promising diagnostic marker for this disorder. This information may have enormous impact in the era of enzyme replacement therapy and thus this condition could be to some extent preventable, once proper genetic counseling and testing for carriers of the mutations in families with this condition is possible. In the present study we aimed to develop and validate a new HPLC method for the detection of S-Ado in the CSF.

Material and Methods

HPLC system with anion exchange Sphere Clone 5u SAX (250 x 4.6) has been developed using synthetic S-Ado in different concentrations to obtain the linear calibration curve.

A total of 23 CSF samples were obtained from 23 patients, 12 females (52.2%), 11 male (47.8%), age ranging between 1-16 years, are processed for measurement of S-Ado using the currently developed HPLC system. All patients were referred to

the Neurometabolic unit at the National Hospital for Neurology and Neurosurgery during the year 2005 with seizures except 2 samples form known ADSL deficiency male patients received as gift from Dr. Keith Hyland (Horizon Molecular Medicine, Atlanta, GA, USA). We considered the presence of RBCs or hemolysis in the CSF as exclusion criteria.

Outcome and Results

A rapid, accurate, in addition sensitive and specific HPLC system with anion exchange SAX column, to detect S-Ado in CSF of patients with ADSL deficiency has been successfully developed and validated. The total time for the test ≤ 15 minutes, and the method validation was achieved by measuring the S-Ado concentration in the CSF of 21 unknown patients presented to the Neurometabolic unit, the observed S-Ado reference range concentration for these cases was found to be varying between (0.55 to 2.36μM). None of the randomly selected disease control cases showed an abnormal S-Ado concentration, whereas the two CSF samples for known cases of ADSL deficiency have shown very high concentrations (356.8 and 357.1 μM) of S-Ado compared with non-ADSL patients. Recovery of S-Ado from spiked CSF after addition of a known concentration (10μM) was achieved by with very high accuracy (range 92%-100%)i.e. greater than 98% in average .The physical properties of S-Ado were found to be stable substance in different and wide range of store temperature i.e. in (-80°C) and also when stored at room temperature (20.8°C), for more than 24 hours .Thus an age related reference range was established for S-Ado in the CSF and the statistical analysis showed no significant cumulative effect of the age or the sex on this reference range. The study was completed within total of 3 month duration, and mean intraday variation was ≤ 5% and the interday variation was ≤ 10%.

Conclusion: the current method is sensitive; specific and enables rapid reliable detection of S-Ado in the CSF of patient with ADSL deficiency in ≤ 15 min.

INTRODUCTION

Adenylosuscinate Lyase (ADSL) deficiency is a rare neurometabolic disease inherited as autosomal recessive trait. The data available reports only sixty cases, but the disease was not reported yet in many countries and many ethnic groups indicating that ADSL deficiency is probably under diagnosed [Kohler et al., 1999].

The disease manifests clinically with psychomotor and growth retardation associated with autistic features of various degrees, convulsions, and muscular dystrophy associated with hypotonia [Van Den Berghe et al., 1997]. Those features are associated and directly related to the accumulation of the dephosphorylated substrates of the enzyme ADSL,which are succinyladenosine (S-Ado) and amino-imidazole succinocarboxiamide riboside (SAICAr) in body fluids [Jaeken and Van Den Berghe, 1984].
These two dephosphorylated substrates are succinylpurines not detectable in cerebrospinal fluid of normal subject and detectable only in ADSL deficient patients and both are products of dephosphorylation reaction mediated by the cytosolic enzyme nucleotidase [Van Den Berghe et al., 1997].
In addition to that, confirmation of enzymatic assays in different tissue including the liver, kidney, and muscles, but not the brain tissue, was all performed previously. All experiments in those assays provided evidence for ADSL deficiency with accumulation of S-Ado and SAICAr [Jaeken et al., 1988;Jaeken et al., 1992].

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Adenylosuccinate (S-AMP) is dephosphorylated by the ADSL enzyme to Succinyladenosine (S-Ado) while amino-imidazole succinocarboxiamide ribotide (SAICAribotide) is dephosphoralated by the same enzyme to amino-imidazole succinocarboxiamide riboside (SAICAr) [Van Den Berghe et al., 1997].

Interestingly, the higher levels of S-Ado were first reported in 1977 in the urine of patients with colon carcinoma; the author attributed these higher levels of S-Ado to relative increase of ADSL within the tumor and recommended its use as neoplastic marker [Chheda, 1977].

The ADSL enzyme plays central and important role in *de novo* synthesis of purines as well as the purine nucleotide cycle [Van Den Berghe et al., 1997].

The role of ADSL in the *de novo* synthesis of purines is to catalyze the nonhydrolytic reaction results in formation of the fumarate from a succinyl group; as well as the phosphorylation of SAICAr into (SAICA ribotide) (Figure 1).

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[Redinbo et al., 1996]

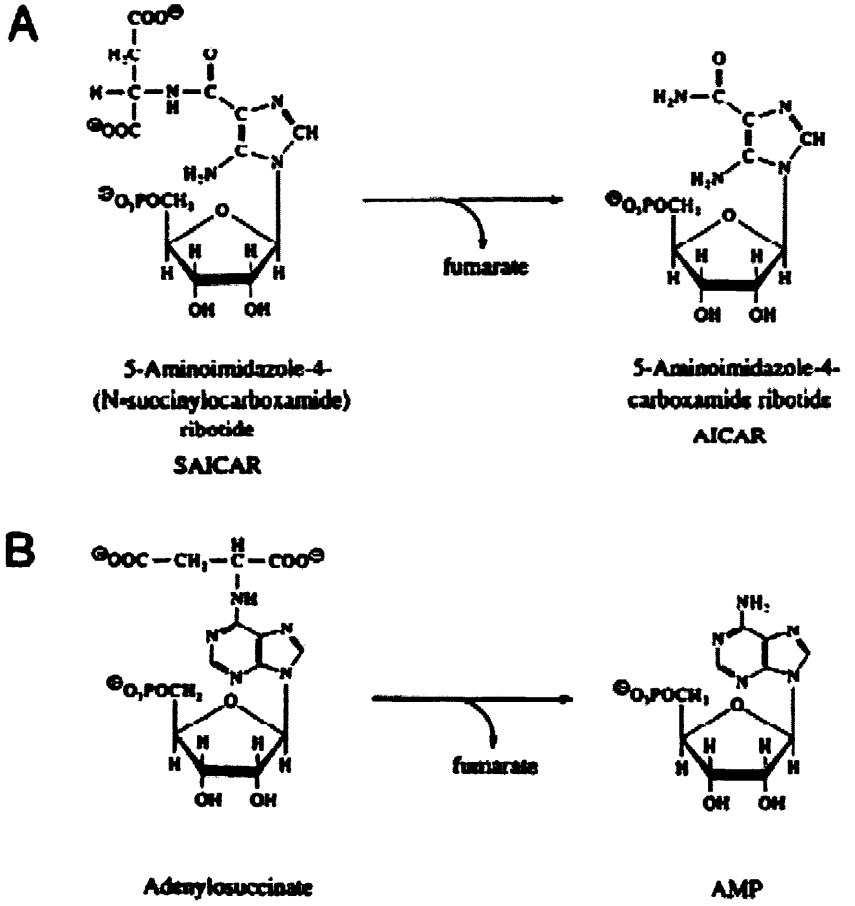

Fig. 1. The two reactions in de novo purine biosynthesis catalyzed by adenylosuccinate lyase (ASL). **A:** Ninth step in the 11-step de novo biosynthesis of IMP from ribose-5-phosphate. **B:** Second step in the conversion of IMP to AMP.

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Taken from Medical Biochemistry: Student Consult with permission from the author.

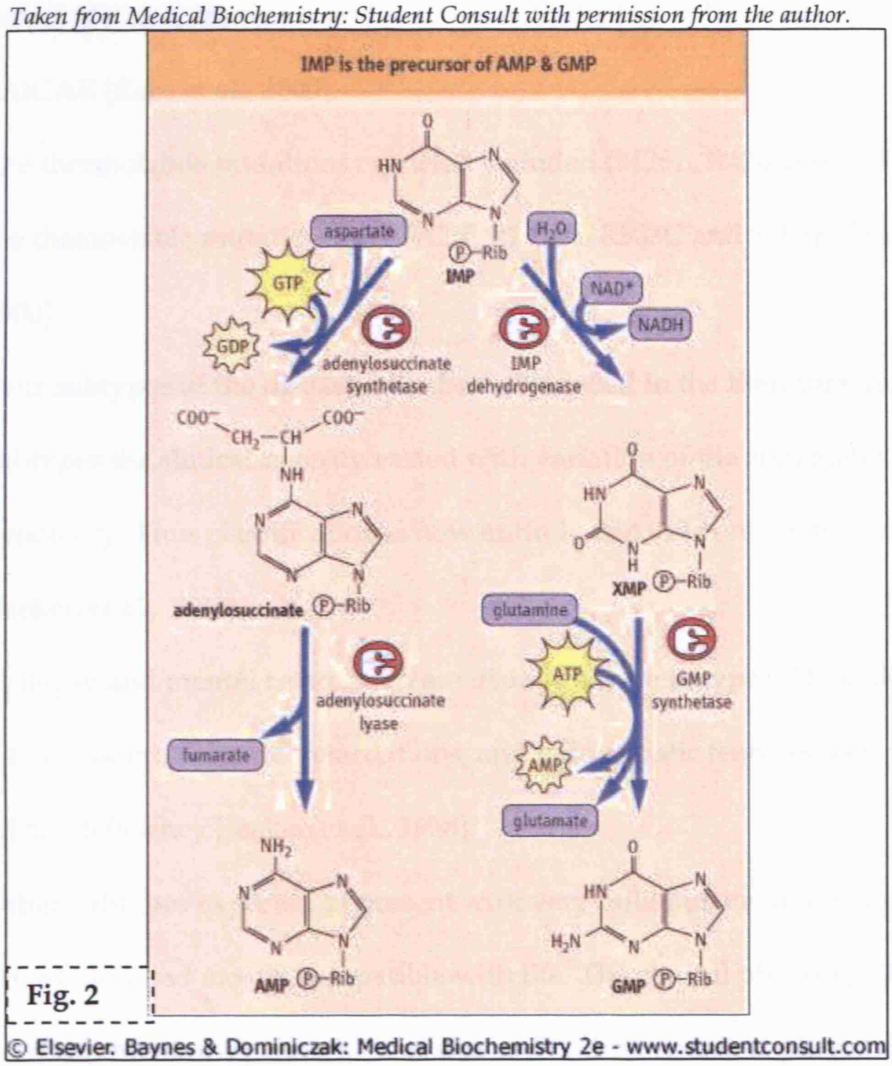

Fig. 2

In contrast to the role of ADSL in the de novo synthesis of purine, its role in purine nucleotide cycle mediate the conversion of S-AMP into AMP, but this step needs the action of the enzymes adenylosuccinate synthetase and AMP deaminase (Figure 2).

In a correlation study between ADSL enzyme mutations and the enzyme activity; about 33% of the mutations were found to be thermolabile, while the majority of the mutations proved to be thermostable and only one mutation

defect (del206-218) was found inactive with the two substrates S-AMP and SAICAR [Race et al., 2000].

The thermolabile mutations reported included (M26L, R426 and T450S) while the themostable mutation were (A2V, R141W, R303C and S395R) [Race et al., 2000].

Four subtypes of the disease have been described in the literature. In these subtypes the clinical severity varied with variation of the enzymatic deficiency. Thus classification is now entirely depends on the disease severity [Jaeken et al., 1988].

Epilepsy and mental retardation are usually severe in type1 ADSL deficiency, while moderate mental retardations, and mild autistic features seen in type2 ADSL deficiency [Jaeken et al., 1988].

Other subtypes expected to present with very mild autism and mental retardation and mostly compatible with life. The clinical phenotype has been correlated with the genotype and also with the biochemical phenotype [van den Berghe et al., 1993].

Although neurotoxicity of the substrates of ADSL enzyme have been suggested and evidenced by experimental animal studies the most probable explanation for the wide variation in the clinical phenotype is the variation in the enzymatic deficiency of ADSL or the residual enzyme activity [Race et al., 2000].

Due to this wide variation in the clinical phenotype it seems that the disease exhibiting an iceberg phenomena and only the tip of the pyramid can be seen;

this warrants screening for patients presenting with seizures, mental retardation or psychomotor delay of unknown cause [van den Berghe et al., 1993].

Although S-Ado is proposed to be a marker for ADSL deficiency, it is not a neurotoxic substance and may has a protective role, as it is detectable in very high concentrations in relatively mildly affected patients [Kohler et al., 1999].

1.1 ADSL Enzyme structural and genetics features

Adenylosuccinate lyase enzyme is a homotetramer bifunctional enzyme, acting in both the de novo purine synthesis and purine nucleotide recycling pathways[Kmoch et al., 2000].

ADSL is a member of metabolic enzymes superfamily known as fumarse enzymes, as all members of this family catalyze reactions with fumarate as one of the products. Other members include aspartase (EC 4.3.1.1), fumarse (EC 4.2.1.2), argininosuccinate lyase (EC 4.3.2.1), δ-crstallin and 3-carboxy-cis, cis-muconate lactonizing enzyme (CMLE; EC 5.51.5) [Yang et al., 2004].

Any member of this family exhibit low sequence homology, but they have strikingly similar tertiary structures[Toth and Yeates, 2000].

The most significant structural difference across the enzyme superfamily seen in domain D1 and D3 while all of them retained highly conserved structure of the core D2.(Fig.3) These structural differences correlate to differences in the substrates of the enzymes superfamily [Toth and Yeates, 2000].

The active site of ADSL is wide so it accommodates its bulky substrates, when compared with the enzyme fumarse C which has smaller substrate so its active site cleft is also smaller[Toth and Yeates, 2000].

The active site of enzyme member of the fumarase family is in close proximity to the signature sequence, which is highly conserved sequence found in residue 284-303 of human ADSL[Kmoch et al., 2000].

The subunits of this enzyme have a molecular weight of 52 KDa; the structures of this enzyme have been determined by using thermotoga marittima and pyrobaculum aerophilum at 1.8 and 2.1A° respectively.

The structural features of ADSL from sapiens, Bacillus subtilis ,T.maritima and P.aerophilum appear remarkably similar [Lee et al., 1999] .

The gene coding for ADSL enzyme consist of 13 exons spaning 23 kb.

Experimental studies showed that ADSL enzyme can expressed from complete ADSL cDNA and the isoform has been produced by alternative splicing by skiping exon 12, interesting only the unspliced form was found to be active[Kmoch et al., 2000].

Adopted from Molecular Genetics and Metabolism 89 (2006) 19–31[Spiegel et al., 2006]

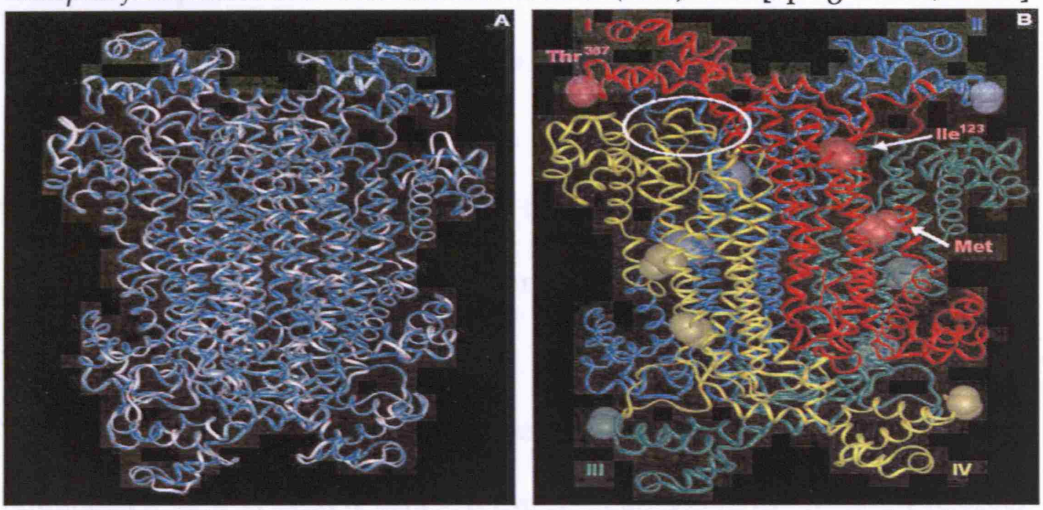

Fig. 3. (A) An overlay of the *B. subtilis* adenylosuccinate lyase and the human enzyme (white) models. (B) Homology model of *B. subtilis* adenylosuccinate lyase based upon the *T. maritima* crystal structure.

ADSL deficiency is the first enzymatic deficiency detected in *de novo* synthesis of purine metabolism [Jaeken and Van Den Berghe, 1984].

The autosomal recessive mode of inheritance has been indicated by the occurrence of the enzymatic defect in two families with consanguinity and more than one child affected of both sexes [Jaeken and Van Den Berghe, 1984].The gene encoding for this enzyme maps to the long arm of chromosome 22 at the region (22q13.1 – 22q13.2) [Van Keuren et al., 1987]; [Fon et al., 1993].ADSL gene contains 13 exons and has approximate length of 21 Kb. The expression of this gene predicted to reveal 484 amino acid protein [Kohler et al., 1999].About thirty-eight mutations have been reported in more than 40 unrelated families and originally from different ethnic groups. Most of them are missense mutations, however substitutions or splice site mutations were all reported [Marie et al., 1999].

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The most frequent mutation in most of the series is (R426H substitution), it has been found in 14 European families of which nine were homozygous ones [Marie et al., 1999].

The mutation (R426H) was reported in three patients and in every one the disease showed severe clinical characteristics form and instability of the enzyme structure [Race et al., 2000].

The first two German mutations reported in ADSL gene were the point mutations (c.423C-T) and (c.620C-A) [Marie et al., 1999]. Interestingly (c.620C-A) mutation occurs in one allele and base pair change in genomic DNA resulted in 39 bp deletion in cDNA and expected to result in a deletion of amino acids 206-218 in the translation process of the enzyme protein composition [Kohler et al., 1999].

There is two exceptional mutations; 39 base pair deletion in the ADSL gene leading to new spilce site and mechanism; and the other is not within ADSL gene but rather a mutation in the promoter region of the ADSL gene and found to be a nuclear respiratory factor 2(NRF-2) binding site[Spiegel et al., 2006]. This mutation has been reported in three patients from one family they were all presented with profound mental retardation and convulsions and it was confirmed that their ADSL enzyme expression was reduced[Spiegel et al., 2006].

There is a correlation between the enzyme activity and the genetic defects suggested by the presence of a relationship between enzyme activity and the ratio of S-Ado: SAICAR in mentally retarded patients [Race et al., 2000].

The variation of the clinical features of ADSL affected families is mostly due to different mutations, resulting in different enzymatic activity; although one substitution mutation (Arg-His) at position 401 has been found in three unrelated patients [Sebesta et al., 1997].

The nucleotide sequence for ADSL gene encodes for 459 amino acids with molecular mass ~ 52 KDa ; human liver cDNA was used to determine this structure [Stone et al., 1992]. Single nucleotide substitution (T - C) resulting in amino acid change (Ser -Pro) at position 413 has been reported in one of the first investigated families with ADSL deficiency [Stone et al., 1992].

Recent recombinant studies in mutant enzyme showed kinetic characteristics similar to those of normal enzyme which denotes that ADSL gene mutations leads in most events to structural problems and excludes catalytic defect [Van den Bergh et al., 1993].

In a correlation study of ADSL enzyme mutations with the enzyme activity about 33% of the mutations were found to be thermolabile , while the majority of the mutations were found to be thermostable and only one mutation defect(del206-218) was found thermally inactive [Race et al., 2000].

The thermolabile mutations reported were (M26L, R426 and T450S) while the thermostable mutation are(A2V, R141W, R303C and S395R) [Race et al., 2000].

The NRF-2 has a suggested role in the regulation of purine biosynthesis; this effect probably exerted through unclear mechanism involving the

transcription of the nuclear genes which is presumably contributing to the mitochondrial function[Spiegel et al., 2006].

1.2. ADSL thermal Stability and catalytic activity

The wild type of ADSL and several mutants were thermally stable at different temperatures below 53°C for at least 60 min [Race et al., 2000].

The thermal stability of the wild type of ADSL also had been investigated in temperatures above 35°C together with the mutant ADSL- S438P which showed thermal stability above 25°C; in addition the mutants A2V and R141W were also found thermally stable [Race et al., 2000].

In contrast the mutant S395R and R303C which are not thermally stable after incubation for 5 min and 60 min consequently ;although showed at least 25% loss of activity [Race et al., 2000].

In correlation study of profoundly retarded patients probably ADSL deficiency type 1 resulted in structural defect leading to markedly decreased enzyme stability ; instead of mild retarded patients with impaired S-AMP binding and catalytic defect suggested to be positive charges effect in the active site [Race et al., 2000].

1.3 ADSL and antiretroviral activity

Additional feature for ADSL enzyme is that it plays an important role in the bioprocessing of anti-HIV therapeutics such as dideoxyadenosine (ddAdo) a selective inhibitor of HIV virus replication through inhibition of virally encoded DNA polymerase (reverse transcriptase) by the triphosphate [Mark,

1987].(ddAdo) was found to be metabolized to its mono, di and triphospate (ddIMP) and affects HIV virus by blocking the cytopathic effect of the virus[Mark, 1987].The main effect of the enzyme ADSL in the (ddAdo) metabolism is indirect through the deamination to ddIno followed by phosphorelation of ddIno to ddIMP then reamination to ddAMP by reaction catalyzed by ADSL enzyme [Mark, 1987].

1.4 ADSL deficiency and disease pathogenicity

The development of symptoms in ADSL deficiency remains to be clarified. Two hypotheses have been suggested; the accumulation of toxic metabolite such as SAICAR and A-AMP then consequently their dephosphorylated derivatives, SAICA-riboside and S-Ado respectively; or the deficiency of purine, especially the adenine nucleotides [Jaeken and Van Den Berghe, 1984]. In mildly affected patients the level of SAICA-riboside in body fluids are not that different from severely affected patients; while S-Ado level is very high even in mildly affected patients [Van Den Berghe and Jaeken, 2001]. SAICA-riboside might be the neurotoxic substance although S-Ado is not neurotoxic but might have another effect antagonizing the toxic effect of SAICA-riboside [Van Den Berghe and Jaeken, 2001].

The neurotoxicity of SAICA-riboside has been suggested and evidenced by direct injection of the substance in rat's hippocampus; one of the studies and recent investigations failed to demonstrate the neurotoxic effect of this substance in cultured rat neurons [Stone et al., 1993;Van Den Berghe and Jaeken, 2001].

The interaction of succinylpurines with membrane receptors using binding analysis technique was also investigated but also given negative results [Van Den Berghe and Jaeken, 2001].

The remaining possibility to be considered is the intracellular accumulation of ADSL enzyme substrates might be toxic to the neurons; and this hypothesis is under investigation now using ADSL deficient model either by using ADSL deficient neuronal cell lines created by knock in and mutation insertion in the murine gene using the most frequent mutation R426H; or over expression of mutated ADSL in mammalian cells and inhibition of ADSL by compounds like adenylophosphonopropionate [Van Den Berghe and Jaeken, 2001].

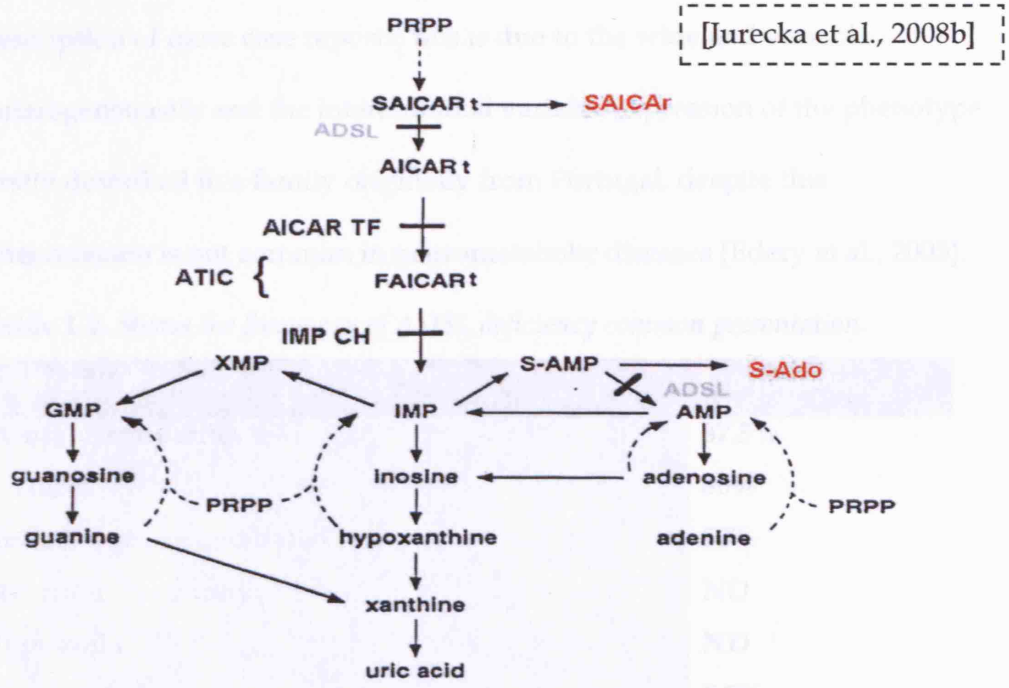


Figure 4. *ADSL deficiency in Purine metabolism, Adopted from A. Jurecka et al. / Molecular Genetics and Metabolism (2008).*

Pathways of purine metabolism

PRPP, phosphoribosyl pyrophosphate; *SAICAR*, succinylaminoimidazole carboxamide ribotide; *AICAR*,aminoimidazolecarboxamideribotide ;*FAICAR*, formyl AICAR; *IMP*, inosine monophosphate; *S-AMP*, adenylosuccinate; S-Ado, succinyladenosine; *AMP*, adenosine monophosphate; XMP, xanthosine monophosphate; *GMP*,guanosinemonophosphate; ADSL,Adenylosuccinate lyase enzyme; AICAR TF, aminoimidazole carboxamide riboside transformylase; IMP CH, inosine monophosphate cyclohydrolase; ATIC, bifunctional enzyme ICARtransformylase/ IMP cyclohydrolase.

1.5 Clinical Features

The first British case reported in 2004 presented at 14 days neonatal age, with a progressive neonatal encephalopathy and seizures. Marked axial and peripheral hypotonia was also detected [Marinaki et al., 2004]. The clinical manifestation of this disease still need to be fully documented with

description of more case reports; this is due to the wide and variable heterogeneousity and the interafamilial variable expression of the phenotype firstly described in a family originally from Portugal; despite this phenomenon is not common in neurometabolic diseases [Edery et al., 2003].

Table 1.1. *Shows the frequency of ADSL deficiency common presentation.*

Clinical feature	Frequency
Mental Retardation	87.5%
Seizures	80%
Autistic feature and behavior	50%
Muscular dystrophy	ND
Hypotonia	ND
Microcephaly	7.7%
Other dysmorphic features	ND

ND: *not determined*

1.5.1 Mental retardation

Mental retardation is the main symptom and described in almost all children with ADSL deficiency, with variable degree including mild to moderate and severe type. The severe degrees of mental retardation were seen in patients with type 1 ADSL deficiency who also present with epilepsy within or after the first year of life [Holder-Espinasse et al., 2002].

Both of the metabolites S-Ado and SIACA riboside have been detected in the CSF of three patients with severe psychomotor delay and autism in high concentrations exceeding 100 micromoles/l [Holder-Espinasse et al., 2002]. Least concentrations of both metabolites were also found in the plasma and urine of those patients; the ADSL enzyme residual activity in one patient

showed absent activity in the kidney and severely reduced activity of the enzyme in liver tissue [Jaeken and Van Den Berghe, 1984].

1.5.2 Autistic features

The autistic features described includes failure to make eye-to-eye contact and gaze impersistance, repetitive behaviour, and agitation with aggression. These features considered one of the common manifestations of ADSL deficiency. Autistic features are seen in 30% to 50% of those children that shows behavioral abnormality considered autistic or semi autistic [Holder-Espinasse et al., 2002]. In further evaluation of those patient showed persistence of autistic feature with little improvement in eye contact [Jaeken et al., 1988].

1.5.3 Dysmorphic features

Although dysmorphic features are not frequently reported in patients with ADSL deficiency, microcephaly, prominent sutures, small nose and brachycephaly were all reported [Holder-Espinasse et al., 2002].
However, microcephaly is rare among patients with ADSL deficiency and seen in about 1/13 i.e 7.7% of all reported cases [Holder-Espinasse et al., 2002].Other Dysmorphic features was firstly described in ADSL deficiency in 2002 ; the case report was female child for unrelated parents she present with Brachycephaly, prominent Sutures, small nose, smooth philtrum, anteverted nostril and thin upper lip [Holder-Espinasse et al., 2002].

The author mentioned that those features could also be seen in patients with other metabolic diseases, mitochondrial diseases, peroxisomal disorder or Fetal Alcohol related toxicity [Holder-Espinasse et al., 2002].

1.5.4 Seizures and Epileptic encephalopathy

The age of onset of seizures in ADSL deficiency is variable range from neonatal period to late infancy and childhood. Nature of seizures in ADSL deficiency also variable including Tonic-Clonic seizures, suppression burst and west syndrome also seen in those children [Holder-Espinasse et al., 2002]. Common feature for seizures in ADSL deficiency is the to resistance all antiepileptic medications [Holder-Espinasse et al., 2002].

1.5.5 Developmental delay and Growth retardation

This feature is seen in some patients but always attributed to feeding problems. Growth retardation showed improvement and some acceleration by the use of adenine (10mg/kg per day) and Alupurinol (5-10mg/kg per day) [Jaeken et al., 1988].

1.5.6 Muscular problems

Both muscular hypotonia and muscular dystrophy are seen in cases with ADSL deficiency [Holder-Espinasse et al., 2002].

This clinical feature can be explained by the skeletal muscle metabolism in which the purine metabolism cycle an unusual feature; it serves to provide the glycolytic intermediates and also involved to replenish the citric acid cycle when the energy demand is high.

The adenine monophosphate (AMP) is converted to Inosine monophosphate (IMP) by deamination. Then (IMP) is converted to adenylosuccinate and the back to AMP (Figure 2).

ADSL deficiency affects this cycle and probably explains the muscular dystrophy but can't explain the hypotonia.

Impaired muscle energy metabolism has been described in one patient with ADSL deficiency this patient presented with mitochondrial dysfunction as a result of reduced muscle energy reserve, in a form of depleted ATP level during and after mild exercise[Spiegel et al., 2006]

1.6 ADSL deficiency diagnosis

1.6.1 Biochemical diagnosis

The basis of this diagnosis depends on presence of the normally undetectable SAICA-riboside and S-Ado in the urine and the CSF of patients with ADSL deficiency.

Because of the variable and heterogeneous clinical presentation of the few number of cases reported with this condition; proper clinical phenotype could only be described by applying wide range of simple and rapid screening program [Van Den Berghe et al., 1997].

The screening program proposed to include all patients with autistic features and mental retardation (psychomotor retardation) and neurological disease.

The biological tests used ADSL deficiency screening depends on succinylpurines detection as marker. It includes Bratton-Marshal test two

dimensional thin- layer chromatography, Silica thin-layer chromatography, capillary electrophoresis high-pressure liquid chromatography with UV detection. [Jaeken and Van Den Berghe, 1984].

Measurement of CSF concentration of Succinylpurines and determination of S-Ado/SAICA-riboside ratio was the trend in some studies [Jaeken et al., 1988].S-Ado/SAICAriboside ratio in severely affected patients is between 1 and 2 ; not like S-Ado/SAICAriboside ratio in mildly affected patient that have S-Ado 5 folds higher; while those with intermediate clinical severity still having ratio between 1 and 2 due to elevated level of compounds in the CSF [Jaeken et al., 1988].Measurement of succinylpurine concentrations in the urine reflects their concentration and/or ratio in the CSF [Van Den Berghe et al., 1997].

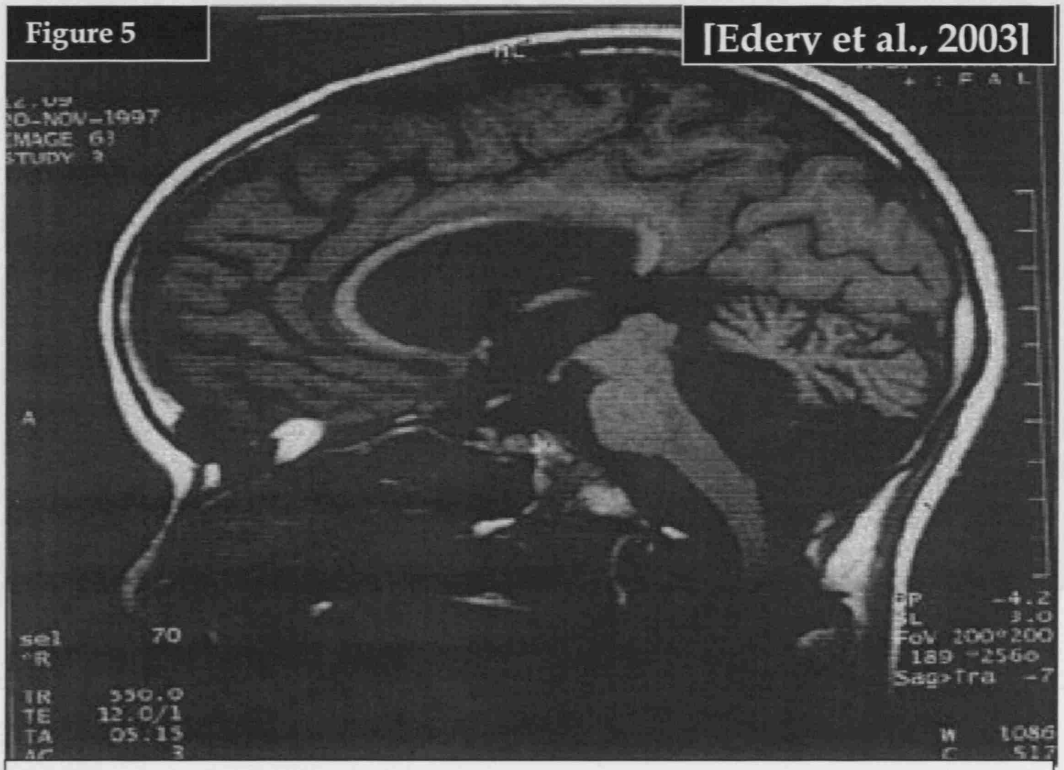
1.6.2 Radiological features

There is group of radiological abnormalities have been reported in ADSL deficiency patients including hypoplasia of the cerebellar vermis , cerebral atrophy, lack of myelination and white matter anomalies including lissencephaly [Edery et al., 2003].

Moreover, PET images for patients with ADSL deficiency diagnosis showed significant results interpreted as diffused profound decrease in the uptake of flourodeoxyglucose (FDG) involving almost all cortical areas; this change was seen in all ADSL deficiency patients included [Spiegel et al., 2006].

The aim of the advanced functional neuroradiology studies was to find evidence for the neurotoxicity hypothesis through structural similarity between the succinylpurine and adenosine A1 receptors; unfortunately in humans, PET scans failed to show decreased re-uptake of FDG in the distribution of adenosine A1 receptors and adenosine re-uptake sites; despite the marked decrease in FDG uptake in all cortical areas using PET scans for ADSL deficient patients. [Spiegel et al., 2006].

Figure 5 **[Ederv et al., 2003]**



Sever cerebral atrophy and cerebellar hypoplasis in T1 MRI image of patient with ADSL deficiency.

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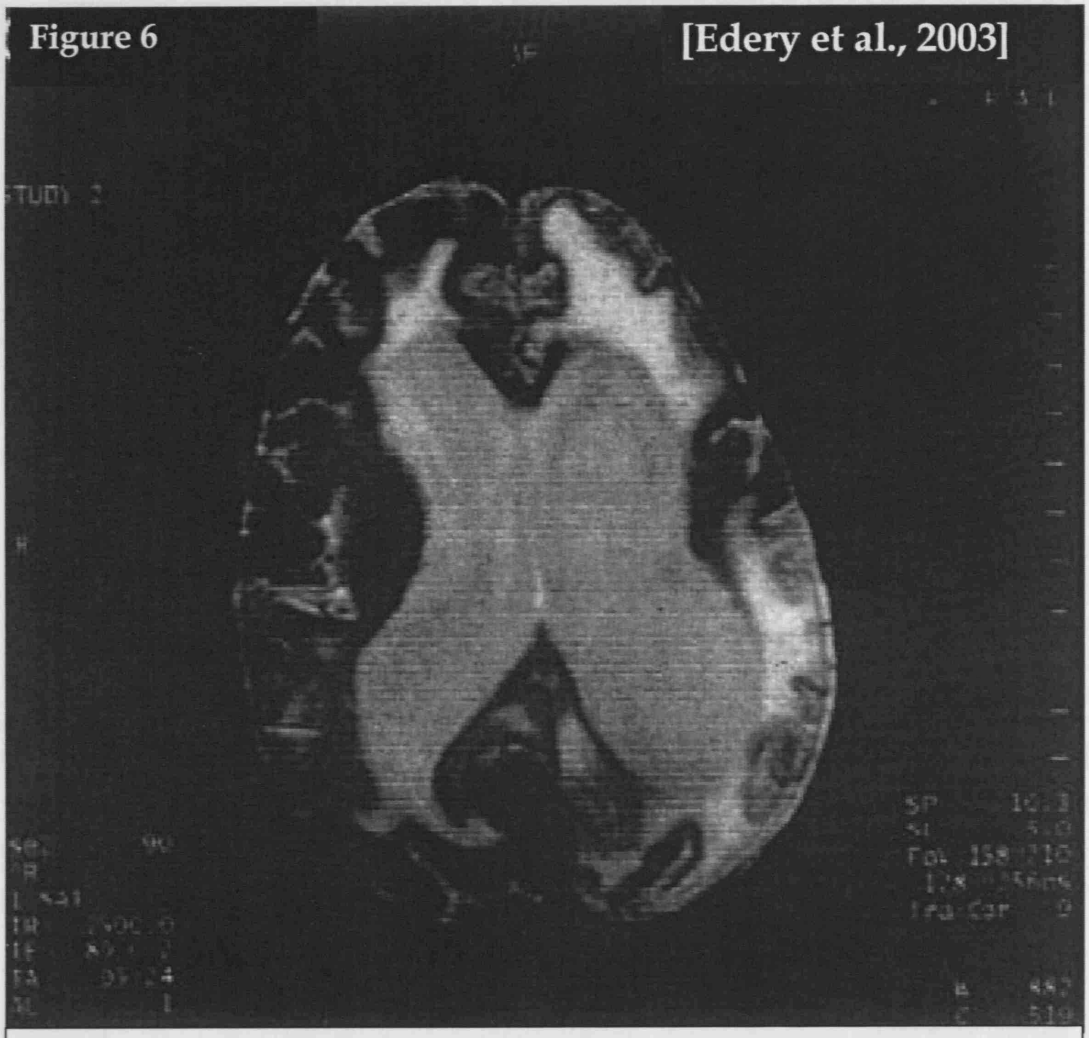
Figure 6 **[Edery et al., 2003]**



T2 weighted MRI showing large area of dysmelinationin in ADSL deficient patient.

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Table 1.2 *Summarizes the charteristics of patients with ADSL deficiency adopted from Molecular Genetics and Metabolism 89 (2006) 19–31*[Spiegel et al., 2006]

Gender	S-Ado/SAICAR	PMR	Autistic features	Other symptoms
M	.98		Severe	Severe hypotonia, convulsions, clonus, cardio-respiratory arrest , death at 6 months.
M	ND	MR	–	Epilepsy, death at 13 years.
F	1.8 (urine)	Present, at 1 year 3 month old, PMD< 6 weeks.	Present, motor restlessness, no eye contact, movements of extremities.	Cerebellar hypoplasia, hypertonicity
F		Severe, at 6 years old, PMD< 6 weeks	Present, stereotyped movements of head and tongue, sensitive to touch	Epilepsy, hypertonicity, thoracic scoliosis, contractures, spastic tetraplegia, severe cerebral and cerebellar hypotrophy
F	0.7	Severe	Blank staring spells	Epilepsy, hypotonia, abnormal cortical function, Lissencephaly.
F	0.9	Severe, lack of motor skills, such as voluntary apprehension and sitting	Severe, no eye contact, lack of language skills, repetitive behavior, stereotypic movements of hands	Epilepsy, microcephaly, muscle hypotonia
M	1.0	PMR	–	Epilepsy, death at 5 months
F	0.9	Severe	–	Epilepsy, apnoea, seizures, coma
F	0.52	–	–	Neonatal encephalopathy, seizures, hypotonia, death at 4 weeks.
F	0.97	Severe, at 27 months old, could not sit unaided and had no speech	–	Hypotonia, seizures, mental retardation, facial dysmorphism
M	0.9	Severe, at 15 months old, PMD at 3–5 months	Mild, washing movements of hands.	Severe muscular hypotonia, epilepsy,

Table 1.2 *Continued*

M	2.4	Mild, at 4 years old, Wne motor and social interactive skills at 3 yearold level	Severe, no eye contact, no social interaction, prefers solo play, early diagnosis of autism	Epilepsy
F	1.2	Severe, standing at 15 months, walking at 2 years	Present, motor restlessness, frequent crying attacks, no eye contact, exaggerated reaction to auditory stimuli, physical agitation of arms and legs, unintelligible speech	Epilepsy, muscle energy metabolism impairmen
M	2.2	Moderate, walking at 2 years, no speaking at 5 years	–	Hypotonia, hyperactivity, erethic oligofrenia, aggressiveness.
F	2.1	Moderate, standing at 1 year, walking at 3 years speaking at 6 years	–	Hypotonia, hyperactivity, temper tantrums, erethic oligofrenia , aggressiveness
M	0.7	Severe, Developmental arrest at 7 months	–	Hypotonia, epilepsy
M	1.5	Severe	–	Epilepsy, apnoea, acidosis, death at 6 months.
F	1.8	Present, sitting at 2 years, walking at 3 years, speaking at 9 years	Present, aggressive behaviour, stereotypes present	

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Table 1.2 *Continued*

F	1.0	Severe, PMD stopped at 9 months	–	Seizures, Death at 15 months
F	1.5	Severe	–	Severe encephalopathy, West Syndrome, hypotonia, epilepsy
F	3.7	Mild, at 4 years old, PMD at 2.5 years	Mild, little eye contact, poor reaction to auditory stimuli	Slow growth (growth improvement with allopurinol treatment
F	1.1	Severe	–	Hypotonia, epilepsy
F	1.6	Severe	–	Hypotonia, epilepsy
F	2.5	Moderate	–	–
M		Moderate	–	–
F	1.5	Severe, at 5 years old, PMD at 6 months	Severe, repetitive activities, grimacing, crying, teeth grinding, biting self, bouts of extreme agitation	Hypotonia
F	1.0 (urine)	Severe, at 20 months old,PMD at 6 months	–	West syndrome, seizures, hypotonia.
F	2.6 (urine)	–	Mild	Profound muscle hypotonia
M	1.2 (urine)	Severe	–	Seizures, hypotonia, deafness
F	1.23	Severe, at 6 years old, could stand, scrawl, and speak few words. Psychomotor regression to bedridden state.	Mild, poor eye contact Severe	epilepsy, strabismus, spasticity, muscular wasting, severe growth retardation.
M	1.35	Severe, walk with aid and say few words at age 2, at age	Severe, no eye contact, abnormal ocular movements,	Epilepsy, spasticity, Xexion contractures of joints

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Table 1.2 *Continued*

		15, can sit and walk few steps with aid.	restlessness, continuous head and trunk rocking, screaming, aggressive tantrums.	
M	1.57	Severe, developmental milestones delayed, not able to stand at 11 years old	Severe, no eye contact, erratic ocular movements, absence of speech, peculiar hand use (chafing of hands in front of eyes)	Past epileptic seizures, spastic paraparesis, brachycephaly
M	1.1	Severe, at 22 months old,PMD at 6 months	Present, no eye contact, motor restlessness, temper tantrums, moving hands before eyes, beating legs on bed, hyper flexion of feet	
M	1.7	Severe, at 7 years old, PMD at 6 months	Severe, wandering gaze, no eye contact, rubbing hands and feet, clapping hands, moving hands before eyes, lying with knees raised, beating back on bed, hours handling same object, laughing to self, temper tantrums Slow growth ,	muscle wasting, epilepsy
F	1.3	Severe, at 5 years old, PMD at 5 months	Severe, wandering gaze, no eye contact, rubbing hands and feet, clapping hands, moving hands before	muscle wasting, epilepsy

Table 1.2 *Continued*

		eyes, lying with knees raised, beating back on bed, hours handling same object, laughing to self, temper tantrums Slow growth,

Unless otherwise noted, S-Ado/SAICAR ratios are from CSF. Abbreviations are as follows: PMR, psychomotor retardation; PMD, psychomotor development; ND, not determined; ADSLdb, ADSL database website, www.icp.ucl.ac.be/adsldb.

1.7 Treatment

The aim of the drug trials in one of the studies is to treat ADSL deficiency by replenishing the decreased concentration of adenine nucleotide in deficient tissue. In this trail adenine nucleotide (10mg/kg per day) and Allupurinol (5-10mg/Kg per day) were used synergistically [Jaeken et al., 1988].

Adenine is used depending on hypothesis that it can be involved in other adenine nucleotides by the enzyme adenine phosphoribsyltransferase (Figure 3) while the use of Allupurinol is assumed to be required for prophylactic prevention of kidney stones from adenine conversion into 2,8-dihydroxyadenine by the action of enzyme xanthine dehydrogenase [Van Den Berghe et al., 1997].

Although some improvement in growth recorded, this trial showed neither clinical or even subclinical improvement nor deterioration in their clinical manifestations.

The most recent therapeutic trial used D-ribose failed to show any clinical

benefits when evaluated in both milder and severe phenotype of ADSL patients[Jurecka et al., 2008]

1.8 Prognosis

The prognosis of ADSL deficiency type 1 proved to be poor, those patients present with lethal fetal intrauterine growth failure, microcephaly, fetal hyperkinesias and in early neonatal period, they need mechanical ventilation to prevent inevitable early neonatal death as they suffer also from intractable seizures [Van Den Berghe and Jaeken, 2001]. The oldest patient reported with ADSL deficiency has reached 20 years of age in addition to two other patients with mild ADSL deficiency died at the age of 8 and 13 years [Van Den Berghe et al., 1997].

2. THE RATIONALE AND OBJECTIVES

2.1 Rationale of the Study

ADSL deficiency is, considered as, a rare cause of childhood epilepsy with only 60 cases reported till now in the literature. In fact this may not represent the whole picture since many believe that the disease is under diagnosed due to difficulties encountered during the diagnosis. In addition to that, the lake of widely available, accurate diagnostic test in communities with high rate of consanguinity has masked the real magnitude of the disease. Most of the previous and recent reports have indicated that the metabolites S-Ado and SIACAr are detectable in the body fluids[Spiegel et al., 2006].

In the present study we aimed to developed and validate a method capable to detect the presence of S-Ado in the CSF of patients.

2.2 Objectives

The main objectives of this project were:

1. To develop and to validate a new method for the detection of (S-Ado) in CSF samples form patients with ADSL deficiency.
2. To validate the method developed by screening patients with features that may resemble ADSL deficiency, such as seizures of unknown underlying cause, psychomotor retardation with or without autistic features in addition to other dysmorphic features.

3. To explore possible sex and/or Age variation in the value of normal range of S-Ado concentration in CSF.
4. To evaluate the stability of the S-Ado in different store temperatures.

3. MATERIALS AND METHODS

3.1 Sample selection

The study included twenty-one CSF samples received by the Neurometabolic unit during the year 2005. The selection criterion for the CSF samples was only random selection for innominated CSF samples, from different sex group and variable age at the recipient time, those sample are archive CSF samples for patients with different neurological presentations. All samples were stored in - 80◦C in the Neurometabolic unit at the National hospital for Neurology and Neurosurgery, Queen square- London-UK. Two CSF samples for confirmed ADSL deficiency patients received as gift from a collaborating lab in the USA.

The presence of RBCs or hemolysis in the CSF is an exclusion criteria for the sample from the study, none of the samples selected showed this.

3.2 Methods

3.2.1 High performance liquid chromatography (HPLC)

HPLC has been the main method for Detection of succinlypurines as specific diagnostic marker for ADSL deficiency diagnosis, reversed-phase HPLC is most widely used in several studies [Jaeken et al., 1988,Van Den Berghe et al., 1997] as will be discussed later in chapter 5.

3.2.1.2 The Current HPLC method development

3.2.1.2.1 Apparatus and instrumentation brief description

Solvent delivery system: a pump (Jasco PU 980), HPLC SAX anion exchange column" Sphereclone 5 U SAX "; injection port; flow-through detector and recorder the role each part is described below. (Appendix 2)

HPLC SAX column is designed to obtain high efficiencies and resolutions, for separation of nucleic acids, organic anions, and is specifically effective for nucleotides. The characteristics and features of the SAX columns will be discussed below.

The current method for detection of S-Ado in CSF HPLC method described below.The general principles for HPLC application will be detailed later in this chapter; with special consideration for the general rules when performing an HPLC test to detect purine metabolites in the CSF.

3.2.1.2.2 Summary of HPLC conditions

SAX column description:" Sphereclone 5 U SAX" (Appendix 1)

"Size = 250 x 4.6 mm"

Wave length detection for S-Ado= 268 nm

Loading time for each sample= 15 min (modifiable).

3.2.1.2.2.1 Testing conditions

The HPLC systems we have used consist of a pump (Jasco PU 980) in which manual injection rather than autosampler is performed and the followings are the testing conditions adopted:

- Flow rate: 0.6 ml/min
- Detection: 254 nM
- Temperature: AMBIENT
- Injection: 1 µl
- Backpressure: 812 psi
- AT(Attenuation time): 8
- Chart Speed: 0.25 Cm/Min
- λ 268 nM Range 0.04

3.2.1.2.2.2 Anion exchange SAX Column specification

Description: Sphere Clone 5u SAX (Appendix 1)

Part No: 00G-4149-E0 Phenomenex

Serial No: 3745056-1

Column size: 250 x 4.6 mm

Column Temperature: AMBIENT

The SAX column has been used in Neurometabolic disorders testing before this current use, for detection of Orotic aciduia, one of the pyramidine metabolic defects [Brusilow and Hauser, 1989].

One of the important features of SAX column is the mixed separation mode, thus it is efficient strong anion-exchanger for inorganic and organic anions.

One of the important features of SAX column is the mixed separation mode, thus it is efficient strong anion-exchanger for inorganic and organic anions[Fifield and Kealy, 2000].

The high anion-exchange capacity can be controlled by the amount of polymer deposited is another advantage[Fifield and Kealy, 2000].

HPLC SAX column is designed to obtain high efficiencies and resolutions, which make it ideal for separation of nucleic acids, organic anions, oligonucleotides, amino acid, and peptides and is specifically effective for nucleotides. This type of columns are strong anion exchanger based on ammonium group (NR3+) and has less pH dependence, as it has a wide pH range from (1-12), which make it unlikely to be PH dependent when compared with other column used in HPLC [Fifield and Kealy, 2000].

In addition, SAX column doesn't shrink or swell as function of anionic strength or organic modifier content of mobile phase, as they are thermally stable up to 80°C this cause different selectivity and high speed separation with lower ionic strength mobile phases, which is very important in the preparation of RNA and DNA sample for further studies [Fifield and Kealy, 2000].

3.2.1.2.2.3 HPLC mobile phase preparation for detection of S-Ado

- Add 1400 ml of Dionized Q water (milli Q H2O) to
- 14.8 g KCl (Batch No 104k0150)-Sigma, UK
- 27.2 g KH2PO4 (Batch No A645725528) - Sigma, UK

3.3 Calibration

In order to establish measurable relation between the curves obtained as a result of wavelength detection in relation to the concentration of S-Ado, Beer Lamber Law has been applied:

$A = \epsilon CL$ or $A \propto C$

Absorbance	A
Molar extinction co efficient	ϵ
The concentration	C
Path length	L

The chromatogram graphic curve in general have two measurable values at peak height and the peak area both can be measured manually or using the computer program connected to the HPLC during each run. In order to establish the calibration curve for this method we used the manual calculation and measurements using different concentrations of S-Ado from a stock solution 200µM given as gift from Dr.Keith Hyland (Horizon Molecular Medicine, Atlanta, GA, USA).The following concentrations were used to obtain a calibration curve from the following dilutions 200, 100, 50 and 25µM. The mean intraday variation was ≤ 5% and the interday variation was ≤ 10%.

Table 3.1 *showing the relationship between the S-Ado concentration and the peak height*

Sample concentration1(µM)	Peak height (Cm)
200 µM	12 Cm
100 µM	6 Cm
50 µM	3 Cm
25 µM	1.5 Cm

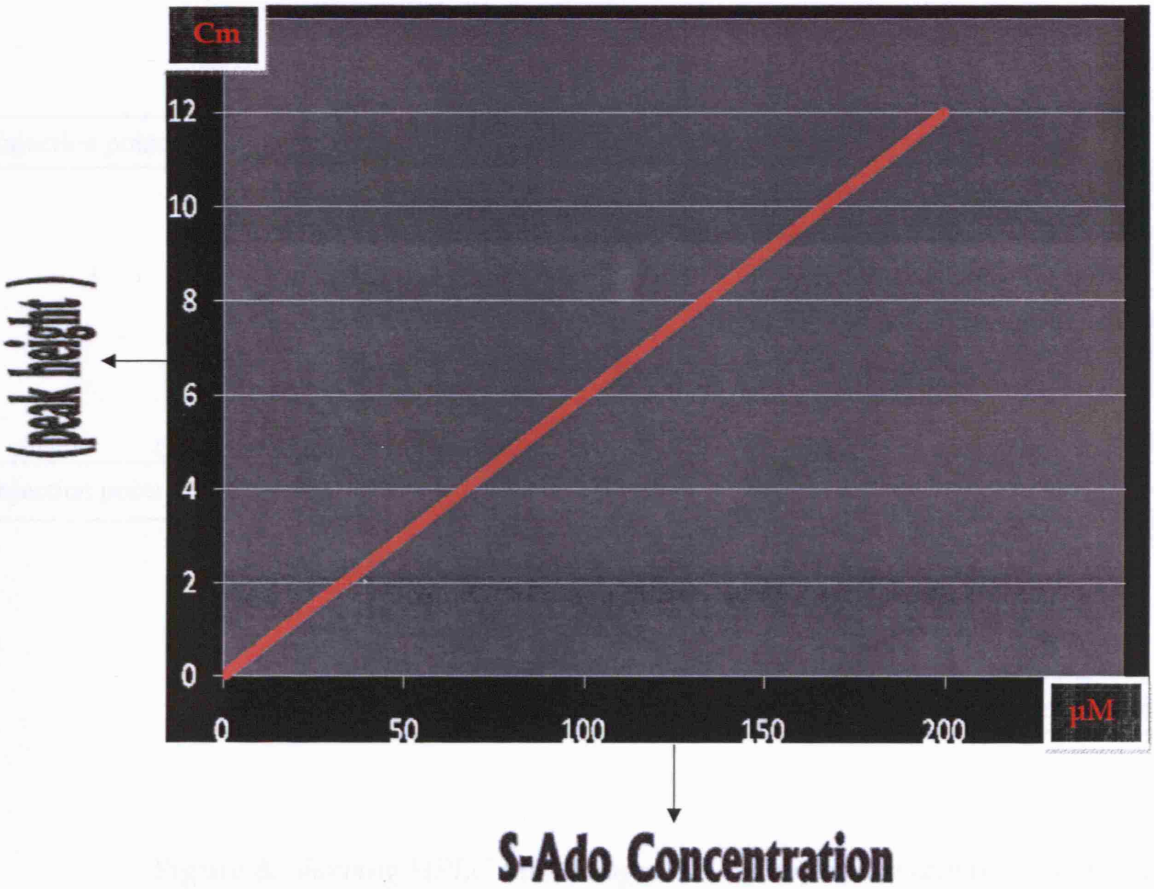

Figure 7. *Shows the linear HPLC Calibration curve.*

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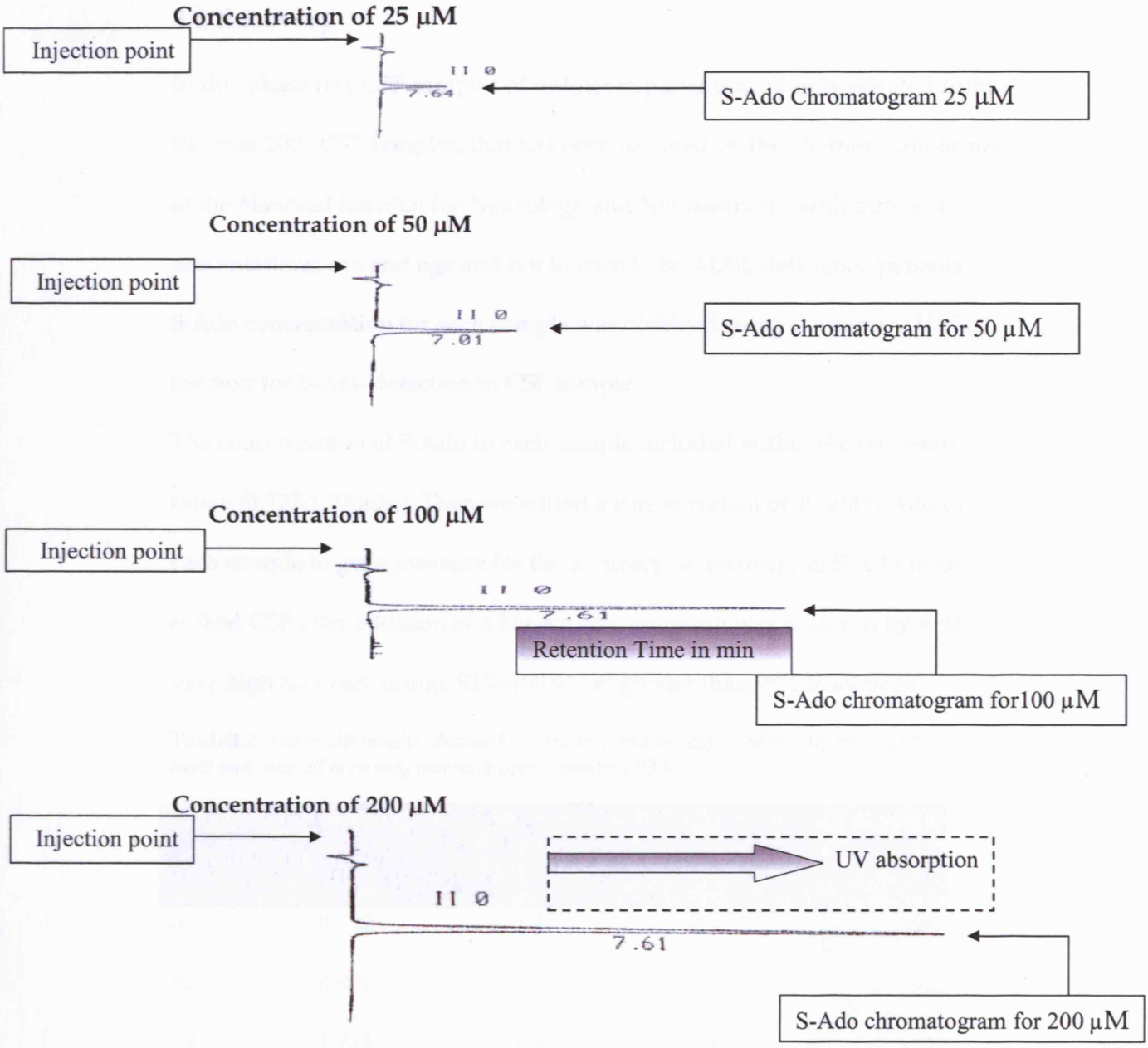

Figure 8. *Showing HPLC chromatograms used to develop calibration curve, as it shows the increase in S-Ado conc. translate into increase in the chromatogram peak height.*

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3.4 Recovery

In this phase five CSF samples of unknown patients randomly selected from the year 2005 CSF samples; that has been received by the Neurometabolic unit at the National hospital for Neurology and Neurosurgery with different presentations, sex and age and not known to be ADSL deficiency patients.

S-Ado concentration for each sample was obtained using the current HPLC method for S-Ado detection in CSF sample.

The concentration of S-Ado in each sample included within the reference range (0.722-1.935μM). Then we added a concentration of 10 μM S-Ado to each sample to get a measure for the accuracy, so recovery of S-Ado from spiked CSF after addition of a known concentration was achieved by with very high accuracy (range 92%-100%) i.e. greater than 98% in average.

Table3.2. *shows the results obtained in recovery and the expected results with confidence limit and over all accuracy reaching approximately 98%.*

CSF sample	*S-Ado conc.*	*Added conc 10 μM S-Ado*	*Expected Final conc.*	*% recovery*
A2	0.722	2.2	2.3	96
B2	0.801	2.2	2.4	92
C2	1.743	3.3	3.3	100
E2	1.935	3.4	3.5	97
G2	0.148	1.7	1.7	100
B5	1.281	2.9	2.88	~100

3.5 Application of high performance liquid chromatography

This method is used in the diagnosis of the majority of the known inherited defects of purine and pyramidine metabolism, and this mostly achieved by applying general screening system involve separation of bases and nucleosides by reversed-phase HPLC and multiwave length UV detection [Duran et al., 1997].

The most appropriate mode choice of HPLC for given separation problems are based on relative molecular mass, solubility characteristics and polarity of compounds to be separated [Denny, 1982].

Reverse phase chromatography using octa decyl (ODS or C18) column and methanol/aqueous buffers or acetonitrile/water mobile phases is by far the most widely used in more than 80% of HPLC systems [Hamilton and Sewell, 1982]. For weakly acidic or basic solutes, pH control is very important as retention times vary considerably with degree of dissociation or protonation [Hamilton and Sewell, 1982].

3.5.1 Disadvantage

Column performance is very sensitive to settling of the packed bed or the accumulation of strongly adsorbed materials or particulate matter at the top; universal detection system not available [Hamilton and Sewell, 1982] .

HPLC has its origins in classical column chromatography, as in column chromatography, the sample is introduced into a liquid mobile phase, which flows through a column of relatively coarse particles of the stationary phase, usually silica or alumina, under influence of gravity[Denny, 1982].

Flow rates are the main determinant factors for the extremely lengthy separation times, as rapid separations would require higher flow rates necessitating the pumping of the mobile phase through the column under pressure [Hamilton and Sewell, 1982].

The poor performance is largely due to very slow mass transfer between stationary and mobile phases and poor packing characteristics leading to a large multiple patch effect [Denny, 1982].

It was recognized that much higher efficiencies and hence better resolution could be achieved through the use of smaller particles of stationary phase, and two basic requirements were developed during 1960 together with suitable pumps, injection systems and low dead- volume detectors and the new technique became known as HPLC [Hamilton and Sewell, 1982].

The mobile phase is typically pumped at pressures up to 3000 psi (200 bar) , and flow rates of 1-5 cm^3 min^{-1} can be achieved by 10-25 cm columns packed with particle as small as 3µm in diameter [Denny, 1982].

A much wide choice of mobile phases facilitates a very considerable variation in the selectivity of separation process. All materials which come into contact with the mobile phase are manufactured from stainless steel, PTFE, Sapphire, ruby or glass for inertness [Denny, 1982].

3.5.2 Mobile phase

In HPLC, appropriate selection of the mobile phase composition is crucial in optimizing chromatographic performance [Gilbert, 1987].

The eluting power of the mobile phase is determined by it's over all polarity. The polarity of the stationary phase and the nature of the sample components. For normal phase separation (polar stationary phase/polar mobile phase) eluting power increases with increasing solvent polarity [Gilbert, 1987].

In addition to solubility parameter and adsorption parameters other properties includes boiling point and viscosity, detector compatibility, flammability and toxicity [Gilbert, 1987].

Generally, lower boiling and hence less viscous solvents give higher chromatographic efficiencies and lower backpressures [Fifield and Kealy, 2000].

3.5.3 The Column

Columns in general are made from straight lengths of precision-bore stainless-steel tubing with smooth internal finish. Typically they are 10-25 cm long and 4-5 mm i.d. microbore columns, 20-50 cm long and with an i.d of 1-2 mm, are some times used where sample size is limited and to minimize solvent consumption because the volumetric flow rate through them is less than a quarter of that through conventional columns [Fifield and Kealy, 2000].

The stationary phase or packing is retained at each end by thin stainless-steel frits or mesh disks of 2µm [Denny, 1982].

Columns are packed by a slurry method, which involves suspending the particles of packing in a suitable solvent and slamming it into the column rapidly and at pressures in excess of 3000 psi (200 bar [Denny, 1982].

HPLC columns need more careful handling and storage to avoid disturbance of the packed bed. They should be kept sealed at both ends when not in use and flushed with methanol prior to sealing [Fifield and Kealy, 2000].

Column life is generally six month or more and can be prolonged by use of a guard and a scavenger column [Fifield and Kealy, 2000].

The former of a very short length of column placed between the injection port and the analytical column to trap strongly retained species or particulate matter originating in the mobile phase, the samples or from wearing of the injection valve [Fifield and Kealy, 2000].

It is packed with relatively large particles (~30 μm) of the same or a similar stationary phase to that used in the analytical column and requires periodic renewal. Scavenger columns are short lengths of tube packed with large particle silica and positioned between the pump and the injection valve with the principle object of saturating an aqueous mobile phase with silica to reduce attack on the packing in analytical column, especially by high or low pH buffers [Fifield and Kealy, 2000].

3.5.4 Sample injection system

Sample may be injected either by syringe or with valve injector although the former is now rarely used [Hamilton and Sewell, 1982].

Valves, which can be used at pressure up to about 700 psi (500 bar). They consist of a stainless steel body and rotating central block into which are cut

grooves to channel the mobile phase from pump to column [Hamilton and Sewell, 1982].

The sample is loaded into stainless steel loop incorporated into the valve body or attached externally whilst the mobile phase passed directly to the column. By rotating the central, block. The flow can be diverted through the loop thereby flushing the sample onto the column [Hamilton and Sewell, 1982]. Returning the block to its original position enables the next sample to be loaded ready for injection. Although the sample injected is generally a fixed volume as determined by the size of the loop, these are interchangeable and range from 2µl to 100µl [Hamilton and Sewell, 1982].Multiport valves which can accommodate several loops of different sizes are available, and some loops can be used partially filled. Automated injection systems are frequently used in industrial laboratories [Hamilton and Sewell, 1982].

3.5.5 Solvent delivery systems

These include solvent reservoirs and inlet filters, solvent degassing facilities and one or more pumps with associated pressure and flow controls. Most systems are microprocessors or computer controlled enabling parameters to be selected and monitored during operation using simple keypad dialogues [Fifield and Kealy, 2000].

The ability to store sets of parameters as modified files and to run diagnostic tests of the system is often available. A single solvent may be used as the

mobile phase for isocratic elution or mixture of two to four solvents [Fifield and Kealy, 2000].

Pumps for HPLC should be capable of delivering a constant, reproducible and pulse free supply of the mobile phase to the column [Fifield and Kealy, 2000].

Constant flow reciprocating pumps are now most widely used type, but because their mechanical action inherently produces a pulsating delivery of the mobile phase the flow must be smoothed so as to eliminate the pulsations[Fifield and Kealy, 2000].

The solvent from separate reservoirs are fed to a mixing chamber via the microprocessor, and the mixed solvent is then pumped to the column. For best reproducibility of solvent gradients small volume pumps (<100µl) are essential [Fifield and Kealy, 2000].

3.5.6 The Detector

The ideal HPLC detector should have these characteristics, rapid and reproducible response to solutes, and a wide range of linear response, high sensitivity and stability of operation [Hamilton and Sewell, 1982].

The most commonly used detectors are based on absorbance of UV radiation and on refractive index.[Hamilton and Sewell, 1982].

Other detectors which are more selective in their response rely on such solute properties as fluorescence, electrical conductivity, diffusion currents and radioactivity [Sewell and Clark, 1987].

4. RESULTS

The currently developed HPLC system and testing conditions for S-Ado detection in CSF were already detailed in the previous chapter.

The experiments on the physical proprieties showed that, S-Ado is stable substance in wide range of store temperatures i.e. -08°C and 20.8°C for more than 24 hours.

The HPLC anion exchange SAX column which has been used in this study separated S-Ado in CSF from salt buffer mobile phase.

The retention time for each sample included was~ (7 -7.5) minutes (Figure 8), and optimal peak shape was achieved for S-Ado in the CSF (Figure 11).

The chromatograms (Figure 13) illustrated the major difference between CSF samples taken from patient with confirmed ADSL deficiency and the disease control CSF samples. Mean recovery was 98% (Table 3.2). In addition, we established an age and gender related reference range for S-Ado in the CSF.

The statistical analysis for the different S-Ado concentrations in the CSF taken from 21 disease control samples patients did not document any significant gender differences on the S-Ado concentration in the various age groups (Table 4.1).

Two patients with confirmed ADSL diagnosis, showed very high concentration of S-Ado in the CSF (356.8 and 357.1 μM) when compared with the newly established reference range obtained from disease controls (0.55-2.36 μM).

Table 4.1 *Shows the statistical analysis for the effect of sex difference on ADSL concentration among the sample population.*

Group Statistics

	Sex	N	Conc Mean	Std. Deviation	Std. Error Mean
conc	Male	8	1.5870	.53958	.19077
	Female	13	1.7492	1.35227	.37505

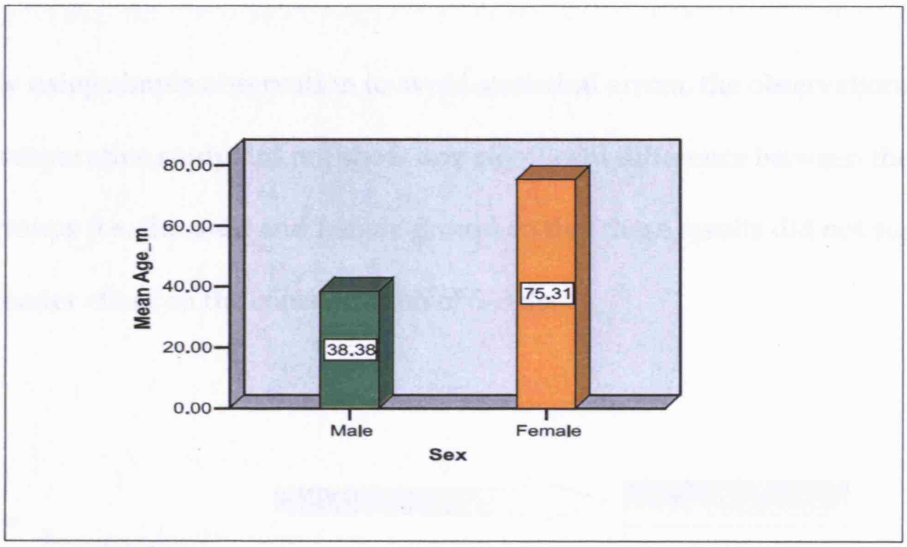

Figure 9. *Shows the mean age (in months) for each Gender group.*

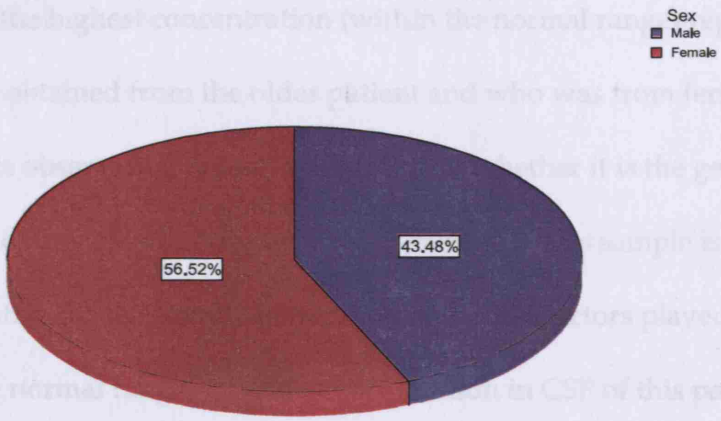

Figure 10. *Shows in percentage the distribution of male and female Gender among the samples.*

By using simple observation to avoid statistical errors, the observational comparative study did not show any significant difference between the two groups (i.e. the male and female group) so that these results did not suggest gender effect on the concentration of S-Ado.

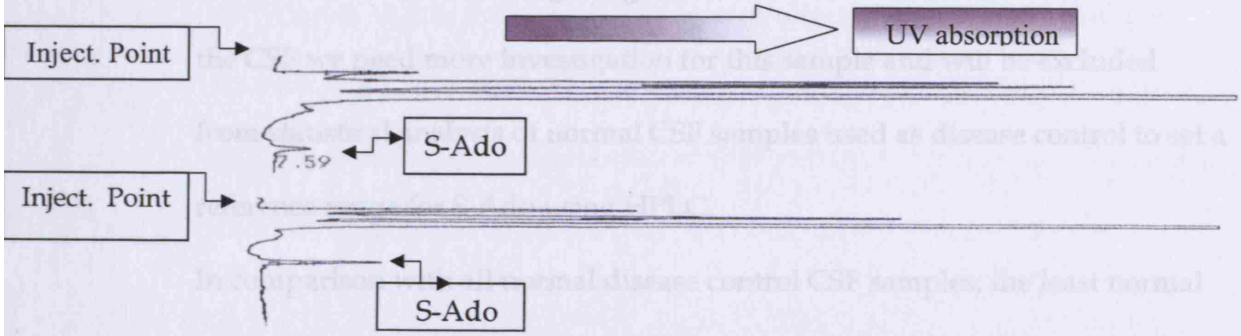

Figure 11. *Shows two HPLC chromatograms for disease control CSF sample.*

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Although the highest concentration (within the normal range) reported in this study was obtained from the older patient and who was from female sex group. This observation failed to differentiate whether it is the gender difference or an age effect (Figure 10). It seems that this sample is not representative for the sample population and other factors played role in raising the normal range of S-Ado concentration in CSF of this patient.

In order to determine the age effect on the reference range for S-Ado concentration in the CSF of the disease control samples. In this study we have taken the CSF samples for patients age between 1 year and 16 years, the results did not show any linear or cumulative effect for age on sample concentration, although the most higher concentration was obtained from a CSF sample taken from 15 year old child; however it seems that is not an age effect because the S-Ado concentration for the most elderly patient among our sample population aged (16 years) from the same sex group (female); did not show the same effect on S-Ado concentration. As we excluded an age or sex effect to be the cause of single highest normal value of S-Ado concentration in the CSF we need more investigation for this sample and will be excluded from statistical analysis of normal CSF samples used as disease control to set a reference range for S-Ado using HPLC.

In comparison with all normal disease control CSF samples; the least normal concentration was not obtained from the youngest patient CSF sample among the sample population of this study; this again emphasis that the age

difference has no role in determining the CSF concentration of S-Ado in normal person or other disease controls (Figure 8).

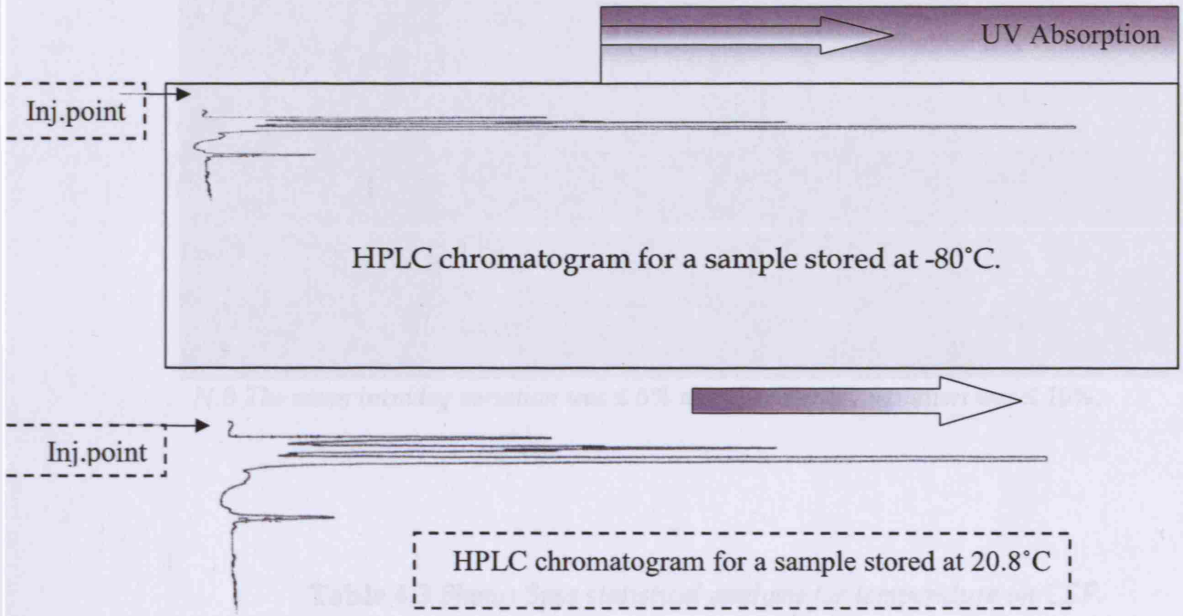

Figure 12. *Shows HPLC chromatogram for the same CSF sample of normal disease control subject in different temperatures.*

The study of S-Ado stability at different extremes of store temperatures, using six samples selected also from different gender (3 males and 3 females) and variable age group, showed that S-Ado is highly thermally stable substance with range of stability varies between 64% and 111% in different CSF samples at store temperature range between -80°C and 20.8°C (Figure 10).

Table 4.2 *Shows reference range for thermal stability of succinyladenosine in room temperature (25°C) for 72 hours.*

Sample	Conc1	Conc2	% of original
C5	0.772	0.815	106
D5	5.895	4.085	64
E5	0.985	1.015	103
F5	1.063	1.089	102
G5	1.81	1.87	103
H5	1.854	2.062	111

N.B The mean intraday variation was ≤ 5% and the interday variation was ≤ 10%.

Table 4.3 *Shows Spss statistical analysis for temperature on CSF.*

Group Statistics

	temp	N	Mean	Std. Deviation	Std. Error Mean
conc	- 80	6	1.8227	1.21521	.49611
	20	6	2.0632	1.92980	.78784

ADSL deficient Patients CSF samples

Two CSF samples were obtained from known males with ADSL deficiency showed extremely high concentration (356.8 and 357.1 µm) of S-Ado; this is confirmative for ADSL deficiency, in addition provided an evidence for the sensitivity and specificity of the newly validated method for S-Ado detection in the CSF.

Clinical feature for those two patients was also very suggestive for this disease; and the clinical data available for us about these two patients is very limited but give hint for ADSL deficiency diagnosis, and subsequent performance of biochemical then genetic testing. One patient presented with seizures of unknown underlying cause presented at the age of 19 month, while the other patient monitored for developmental delay, regression and MRI features suggestive of white matter changes and dysmylination; all these features have been reported else where in different case report.

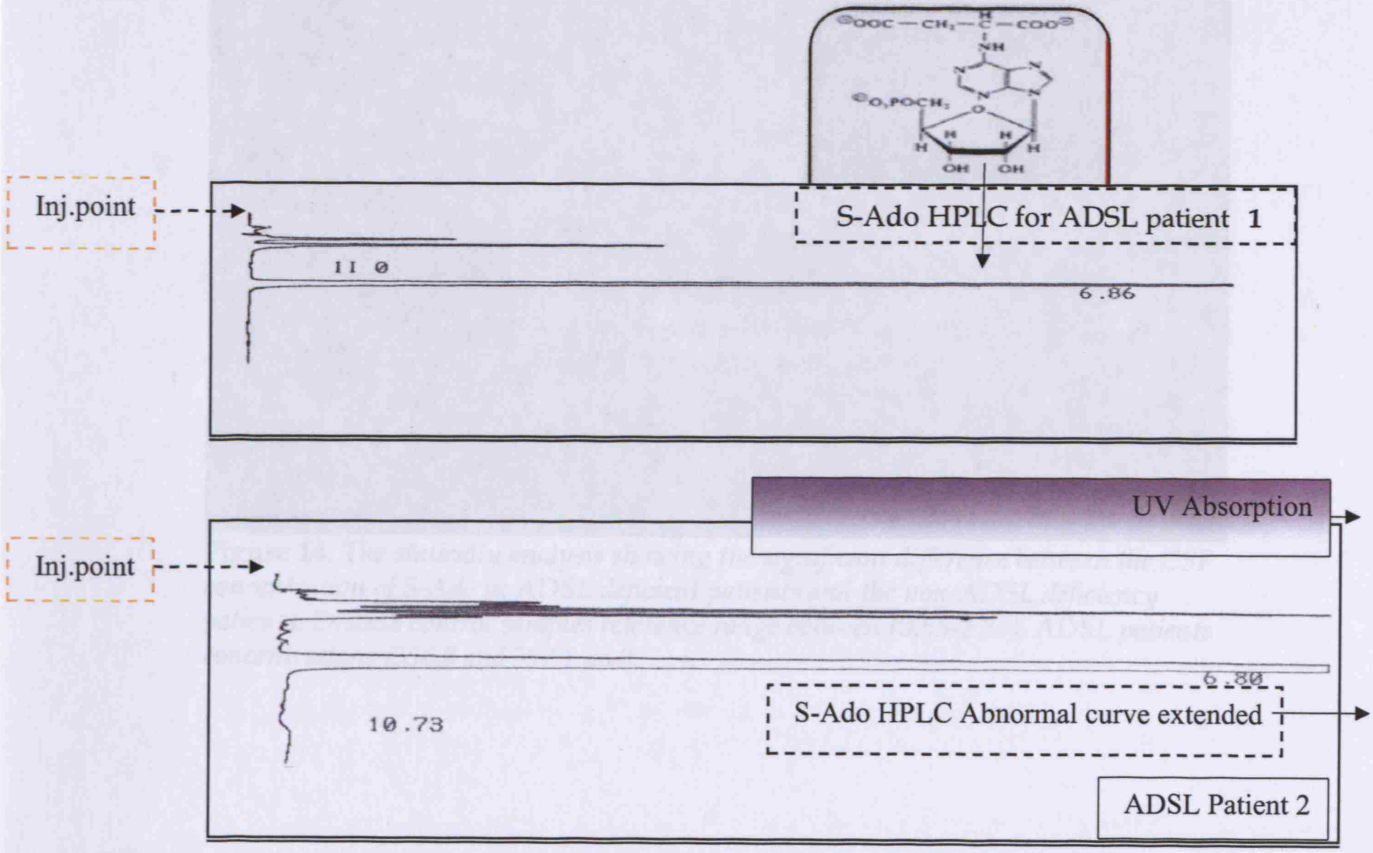

Figure 13.*Shows abnormal HPLC chromatograms for two patients with ADSL deficiency.*

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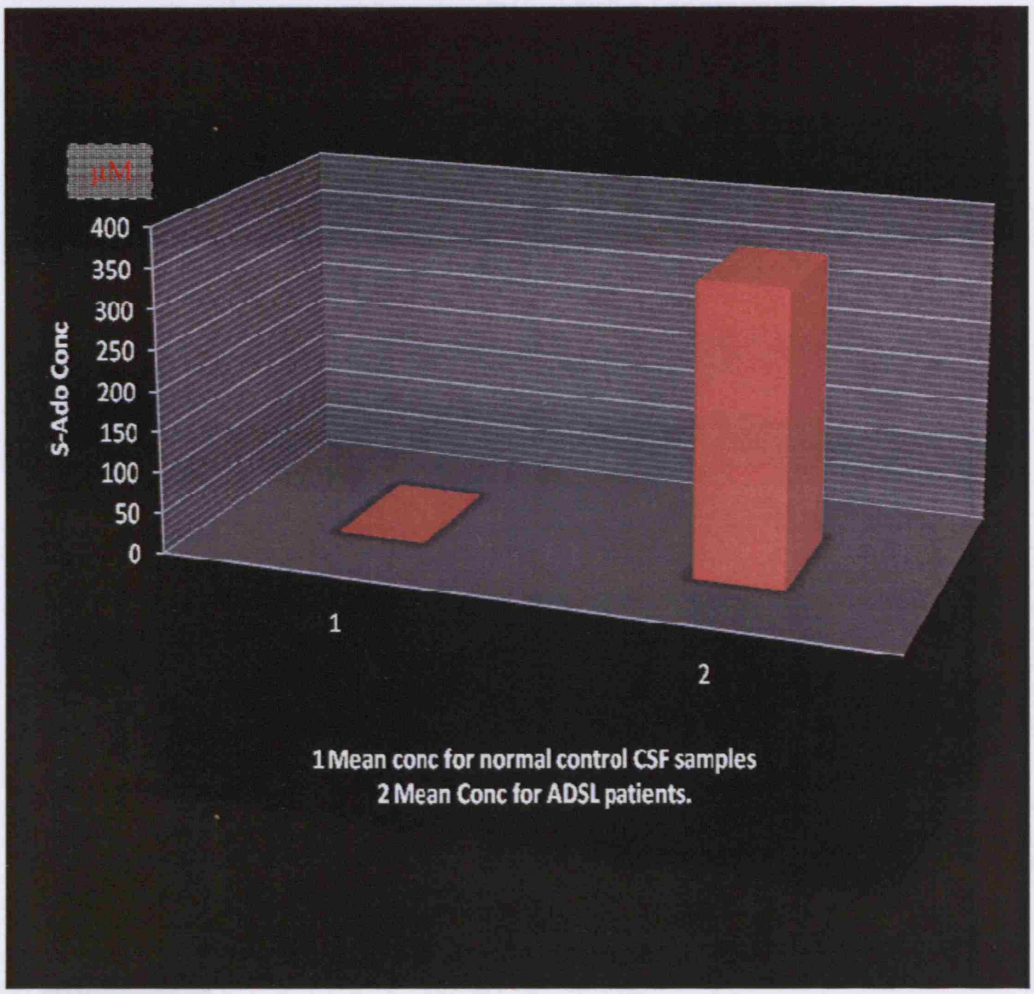

Figure 14. *The statically analysis showing the significant difference between the CSF concentration of S-Ado in ADSL deficient patients and the non-ADSL deficiency patients. Disease control samples reference range between (0.55-2.36), ADSL patients concentrations (356.8 and 357.1 µM).*

Table 4.4 *shows the exact S-Ado concentrations obtained during this study from the disease control sample and the two ADSL deficient patients.*

Sample code	Age/Month	Gender	S-Ado conc. In CSF µM
A2/10	14	F	0.922
B2/10	21	F	0.701
C2/10	61	M	2.143
E2/10	73	M	2.355
G2/10	18	F	0.548
B5/10	16	M	1.281
C5/10	108	F	0.772
D5/10	180	F	5.895*
E5/10	32	M	0.985
F5/10	24	M	1.063
G5/10	48	F	1.81
H5/10	14	F	1.854
A2/11	64	M	1.132
B2/11	192	F	2.063
C2/11	40	F	1.673
D2/11	180	F	1.91
E2/11	15	M	1.722
F2/11	84	F	1.713
G2/11	39	F	1.095
H2/11	30	M	2.015
I2/11	63	F	1.783
2202/ADSL patient.	19	M	356.8
2635/ADSL patient.	46	M	357.1

**This sample excluded from statistic analysis.*
N.B The mean intraday variation was ≤ 5% and the interday variation was ≤ 10%.

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5. DISCUSSION

The metabolism as well as the biosynthesis of the Succinlyadenosine S-Ado is regulated by two enzymes; Adenylosuccinate lyase (ADSL) and Adenylosuccinate synthetase. In patients with ADSL deficiency, the S-Ado appears to be formed in the CSF by dephosphorylation reaction involving the intermediate of purine metabolism Succinyladenosine phosphate and more commonly termed adenylosuccinic acid [Chheda, 1977].

The adenylosuccinic acid is a by product of both salvage and *de novo* path ways of purine metabolism. It is formed by conversion of Inosine Monophosphate (IMP) through a biological reaction with aspartic acid. This is an important reaction, mediated and regulated by the enzyme adenyloscuccinate synthetase and GTP. The adenylosuccinic acid is then cleaved by the enzyme ADSL resulting in formation of AMP and fumarate.

The normal range of S-Ado in CSF of unaffected individual varies between 10 - 100 mumol/l in most series, which is detectable by HPLC, in contrast to the low concentrations found in the plasma and urine of normal individual(5 to 10 mumol/l) [Jaeken and Van Den Berghe, 1984].

Screening for the majority of the known inherited defects of purine metabolism can be achieved by analyzing urinary excretion profiles. The quantitative measurement of urinary uric acid /creatinine ratio is first approach for almost all purine metabolism defects, except two enzymatic deficiencies, i.e. ADSL and adenosine monophosphate deaminase

deficiencies, as they are not associated with changes in uric acid levels [Duran et al., 1997].

Adenosine monophosphate deaminase can not be diagnosed using body fluids, as it manifests only intracellular changes in nucleotide contents. In contrast, ADSL deficiency diagnosis is mainly based on the detection of the enzyme substrates (succinylpurines) in body fluids, and there are several chromatography methods available, including many simple but effective thin layer chromatography(TLC),reversed- phase HPLC system and frit-fast atom bombardment-mass spectrometric method (frit-FAB-MS) [Duran et al., 1997]. TLC is a simple screening technique for detection of ADSL deficiency, but it has many perquisites necessitate manual effort and more time consumption, as the extensive purification of urine step prior to the analysis is very crucial and carries serious draw back on the procedure. In addition, most of the studies used this technique needed to include both substrates(S-Ado and SAICAr) so as to show the quantitative difference between mild and severe cases [Duran et al., 1997].

Reversed- phase HPLC system has been the main method for detection of ADSL in the last two decades; A Spherisorb S-5 ODS1 (25 cm x 4.6 mm) column has given an excellent performance and was used for urine profile in ADSL patients screening studies[Jaeken et al., 1988,Van Den Berghe et al., 1997].

The most recent study in the Neurometabolics screening for both purine and pyramidine disorders Methods, is the introduction of a reversed-phase HPLC

electrospray ionization tandam mass spectrometry, as it is able to detect 24 metabolites for purine and pyramidine metabolism in the urine in one analytical run, thus sensitive and specific for 13 defects of purine and pyramidine metabolism, including ADSL deficiency by detection of S-Ado in the urine [Hartmann et al., 2006].

Although recovery of a compound after addition of known concentration in the previously mentioned study (85%-113%), is near to that obtained in our study (92%-100%) (see table 3.2), the method presented in the current study carries more than three advantages when compared with the former, as it adds a reference range for ADSL metabolites in CSF rather than urine, and this obviously make it more sensitive and specific for ADSL diagnosis, more over less expensive and even by far less time consuming.

Interestingly this recent study [Hartmann et al., 2006], failed to detect SIACAr by using reversed-phase HPLC electrospray ionization tandam mass spectrometry, the author suggested that SAICAr was not ionizable or became instable in the ion source , and alternatively recommended the detection of SAICAr by modified Bratton -Marshall test [Hartmann et al., 2006].

In addition, the reverse-phase column which has been used in the previous study (Aqua C18 Mini-bore; 250 X 2.0mm), unlike the SAX HPLC column which we have used in the current study, as it is very dependent on temperature and pH [Hartmann et al., 2006].

The HPLC SAX column is anion exchange column as described in chapter 3, designed to obtain high resolution and efficiencies wide mixed separation

mode, and wide pH range, which make it best option for nucleotide detection, it is firstly used by Bursilow and Hauser, as they have described Whatman Partisil 10 SAX column (250 X 4.6 mm), and it has given an excellent separation of orotic acid in hereditary orotic aciduria, then subsequently a similar anion exchange column applied for detection of intercellular nucleotides [Simmonds et al., 1991].

To the best of our knowledge HPLC SAX column "Sphere Clone 5u SAX" (250 X 4.6 mm), which we have used in the current study is the first time to be introduced for detection of ADSL and there is no specific study focused on setting a reference range for S-Ado in the CSF. In this view, the current study have been developed using an HPLC system described in chaper 3 for detection of S-Ado in CSF .In addition, this method is sensitive enough to identify patients with ADSL deficiency.

Although the highest concentration (within the normal range) reported in this study was obtained from a relatively older female patient, when compared with the other study population ages; this observation couldn't differentiate certainly whether, it is the gender or an age confounding effect. However, it seems that this sample is not representative for the sample population and other factors elevated the range of S-Ado concentration in the CSF of this patient; this need to be more clarified not necessarily in the context of this study.

In order to determine the age effect, so as to set a reference range for S-Ado concentration in the CSF, disease control samples were used. In this study the

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CSF samples was previously obtained form patients with age ranging between 1 year and 16 years; the oldest age in this study is relatively near to the age of the oldest patient ever reported up the time of this report writing. Although the highest concentration (5.8 μM) was obtained from a CSF sample for a 15 year old child (table 4.4), the results and the statistical analysis did not show any linear or cumulative effect for the age on the concentration of S-Ado in CSF. This can be supported by the fact that S-Ado concentration in CSF for the oldest patient among our sample population in this study has almost the near age (16 years) and has the same gender group, and yet she did not show the same incremental effect on S-Ado concentration. In comparison, the least concentration of S-Ado in CSF was not obtained from the youngest patient sample among the sample population in the study. This also supported the hypothesis that the age has no role in determining the CSF concentration of S-Ado in normal person or other disease controls (Figure 9).

In addition ,we excluded any gender effect may disqualify the respective sample from the statistical analysis of normal CSF samples, and considering all normal disease control CSF samples.

The experiments on S-Ado thermal properties revealed that this substance is thermally stable at least in the room temperature of (20.8°C). This finding is crucial for establishing CSF diagnostic test that dependents on the concentration of S-Ado as the transport of sample and the time factor plays central role in successful test and accurate result especially in hot climate countries , where this test suits the prevalent autosomal recessive inheritance.

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In contrast to the results mentioned above, about the of stability S-Ado in wide temperatures range (20.8°C to -80°C), SIACA riboside is unstable metabolite at room temperature and the sample store in -20°C is mandatory to perform any practical measurements, as in the modified Bartton-Marshall test [Laikind et al., 1986].

This thermal property gives S-Ado an important advantage to be considered and make it superior, when trying to standardize an assay for diagnosis ADSL deficiency, without technical errors and sample store and transport difficulties .Although SIACAr is suggested as possible or probable neurotoxic substance in one of the studies, this neurotoxicity theory has never been proved yet as discussed earlier.

According to the results in chapter 4, detection of high concentration (more than 10 µM) of S-Ado in CSF is very significant marker for ADSL deficiency when correlated with clinical finding, and can be used in patient with wide range of clinical presentations.

Based on this study and considering the previous studies we suggest the use of the currently developed, rapid diagnostic method for all patients presenting with seizures of unknown etiology, with or without mental retardation, autistic feature, and hypotonia with muscular dystrophy and dysmorphic features in order to exclude ADSL deficiency using standardized clinical criteria for patient screening, a recent report also suggested exclusion of ADSL deficiency in any neonatal seizures [Clamadieu et al., 2008].

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Interestingly, a recent single report added more concern for CSF screening of ADSL metabolites using HPLC, as it has documented elevated S-Ado and SAICAr in CSF but not in the urine, taken from a patient diagnosed as fumarase deficient ; the author postulated the cause of an elevated ADSL substrates in the CSF of this patient is due to the excess fumarate has inhibited ADSL enzyme leading to accumulation of SAICAr and S-Ado ; this finding may decrease the specificity of both markers [Zeman et al., 2000].

The most recent reinforcement for the study goal i.e. the use of S-Ado concentration in the CSF as a sensitive diagnostic marker for ADSL, is the postulation of the two substrates (S-Ado and SAICAr) are extra cellular intermediates, so can be detected in body fluids, while the toxic effect is mainly results from the intracellular phosphorylated intermediates (SAICAR and AMPS) when they accumulate inside the cell, this could exert the neurotoxic effect and then perhaps dephosphorylated so as to exit the cell, and subsequently their presence can be confirmed in body fluids especially in large amounts in CSF [Spiegel et al., 2006].

The main aim for most of the previous studies efforts as our current objective, is the development of rapid method for ADSL deficiency diagnosis, by the detection of its substrate S-Ado in the CSF, the largest previous study in this aspect has screened 10,000 urines for S-Ado and SAICAr,surprisingly no case was detected, thus the author considered ADSL deficiency a very rare condition in UK [Marinaki et al., 2004].

In comprehensive review for the previous cases reported with ADSL diagnosis, we did not find any formal consensus for the biochemical diagnostic assays that should be used for ADSL diagnosis, as the first U.S case was detected by high resolution TLC, and then confirmed using reversed phase HPLC as a quantitation method for succinylpurines in the urine, using A Supelco LC-18-S 'nucleoside' column thermostated to 27°C [Valik et al., 1997]. On the other hand, the first British case was reported in 2004, the biochemical diagnosis was performed in urine, plasma and CSF using RPLC (Reversed- phase liquid chromatography) with inline iodide detection. The separation of nucleosides and bases achieved by using Hichorm ODS-1 analytical column [Marinaki et al., 2004]. This method is more time consuming and dependent on pH. While the most recent ADSL case reported in 2007 in Poland, by identification of high amounts of S-Ado and SAICAr in CSF using HPLC with UV-VIS detection and confirmed by TLC and spectroscopy Methods[Jurkiewicz et al., 2007].

In addition, case reports did not show a well defined correlation between the clinical phenotype and the genetic mutations. (Table 1.2) In comparison, the fibroblast activity of the ADSL enzyme deficiency which should be considered after the recent fumarte deficiency case report mentioned above, in spite of the fact of being not correlated with S-Ado: SIACAr ratio and so it does not necessarily always reflecting the ADSL Substrates ratio in the CSF of those patients [Race et al., 2000].

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while, the only well recognized association was seen between the disease severity and the ratio of (S-Ado/SAICAr),this ratio is a round ~ 1 in patient with severe psychomotor retardation while higher ratios (2-4) are detectable in patients with mild phenotype, obviously this ratio depends on both (S-Ado/SAICAr) and inversely correlated to S-Ado level which make this measure of less value and importance when we develop a reference range for S-Ado in diseased subjects [Van Den Berghe and Jaeken, 2001].

In addition, SIACAr is by far thermolabile substance and can be detected in minimal amounts, when compared with S-Ado in mildly affected patients, thus SIACAr concentration was not investigated in this study. On the other hand, S-Ado concentration appears to be more sensitive than measurement of SAICAr; as in mildly affected patients the level of SAICAr in body fluids are not that different from severely affected patients; while S-Ado level is very high even in mildly affected patients [Van Den Berghe and Jaeken, 2001]. This was interpreted by some authors as an indication for SAICAr as neurotoxic substance in contrast to S-Ado which is not neurotoxic and might have another effect antagonizing the toxic effect of SAICAr [Stone et al., 1993;Van Den Berghe and Jaeken, 2001]

However, the recent investigations failed to demonstrate the neurotoxic effect of this substance in cultured rat neurons; this also in favor of using S-Ado as a marker [Stone et al., 1993;Van Den Berghe and Jaeken, 2001].

In conclusion: this study can be considered as pilot study and also added a new rapid technique takes a total of only 15 min or (even less adjustabl time)

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for detection of the CSF marker S-Ado to diagnose ADSL deficiency; although this method is highly sensitive and very specific for this condition; clinical researchers should consider the range of the clinical syndrome spectrum which is variable and wide, includes seizures of different type and severity, mental retardation extremes from mild to severe degree with or without the other clinical features such as the autistic features, microcephaly and hypotonia, in addition to the other dysmorphic features.

The age range for patients that should be offered this diagnostic test is also to some extent wide, as it varies from the neonatal period when the clinical suspicion is high in the presence family history of similar condition; through childhood to adults in early twenties, with mild phenotype including seizures of unknown aetiology and/or mild mental retardation.

The advantage of this method using the rapid detection of S-Ado as fast CSF marker for ADSL deficiency; is the sensitivity and the specificity in addition to the store temperature range of stability that makes S-Ado superior over the other metabolic marker SAICAr.

The other great advantage for testing S-Ado concentration in the CSF rather than SAICAr, is being detectable in large amounts in body fluids, when the patient present with mild symptoms as it has been suggested to have protective effects against neurotoxicity; this fact also could be added to the sensitivity of this marker in clinical consideration of mild phenotype and also applicable on a screening project for this condition.

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On the light of the accurate results using anion exchange SAX column, and mean recovery (98%) in the Neurometabolic laboratory at the National hospital for Neurology and Neurosurgery; beside the availability of the newly established reference range for S-Ado in CSF, we recommend further larger study considering mainly CSF concentration of S-Ado and using the currently presented HPLC Method; as screening and then as primary diagnostic test for all patients presenting with any feature suggestive of ADSL deficiency or in the context of the clinical provisional differential diagnoses.

The enzymatic studies such as, the ADSL enzyme fibroblast activity and common genetic mutation screening should be considered only when high S-Ado concentration in the CSF is confirmed using HPLC as they are more time consuming and very expensive and not widely available in diagnostic laboratories.

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Abbreviations List

ADSL	Adenylosuscinate Lyase
AICAR	Aminoimidazolecarboxamideribotide
AMP	Adenosine monophosphate
cDNA	Complementary DNA
CSF	Cerebrospinal fluids
IMP	Inosine monophosphate
FAICAR	Formyl AICAR
FDG	Flourodeoxyglucose
GMP	Guanine Monophosphate
HPLC	High performance liquid chromatography
KDa	Kilodalton
NRF-2	Nuclear respiratory factor 2
PET	Positron Emission Tomography
PRPP	Phosphoribosyl pyrophosphate
RPLC	Reversed phase liquid chromatography
S-Ado	Succinyladenosine
SAICAR	succinylaminoimidazole carboxamide riboside
TLC	Thin Layer Chromatography
S-AMP	Adenylosuccinate
UV	Ultra Violet light
XMP	xanthosine monophosphate

Appendix 1
HPLC SAX Column

phenomenex
...breaking with tradition

HPLC Certificate of Quality Assurance

Part No: 00G-4149-E0
Description: SphereClone 5u SAX

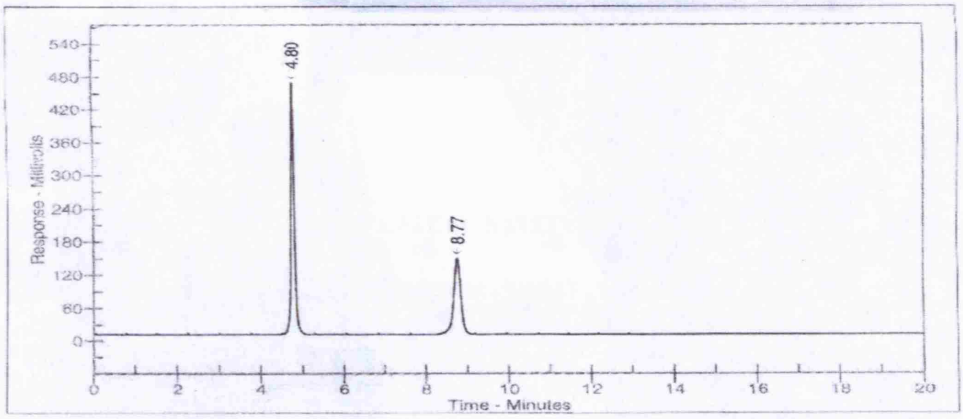
Serial No.: 374506-1
Column Size: 250 X 4.6 mm

TESTING CONDITION

Mobile Phase: 0.15M (NH4)H2PO4, pH 6.0
Flow Rate: 0.6 ml
Detection: 254 NM
Temperature: AMBIENT
Injection: 1 µl
Back Pressure: 812 psi

TEST SAMPLES

1. URIDINE
2. UMP



Peak	Time	Area	Factor	Width	Eff.	Res.	Asym.
1	4.80	2553255	0.000	0.078	20867	0.000	0.95
2	8.77	1637679	0.828	0.173	14114	18.622	0.94

MEASURED PARAMETERS BASED ON UMP PEAK

Efficiency: 56456 p/m
Peak Asym.: 0.94
k Factor: 0.828
Ret. Time: 8.77 min

Instrument: QC43 374506-1
Checked By: KH
Tested: Thursday, December 21, 2006
Approved By:
Printed: Thursday, December 21, 2006 ms

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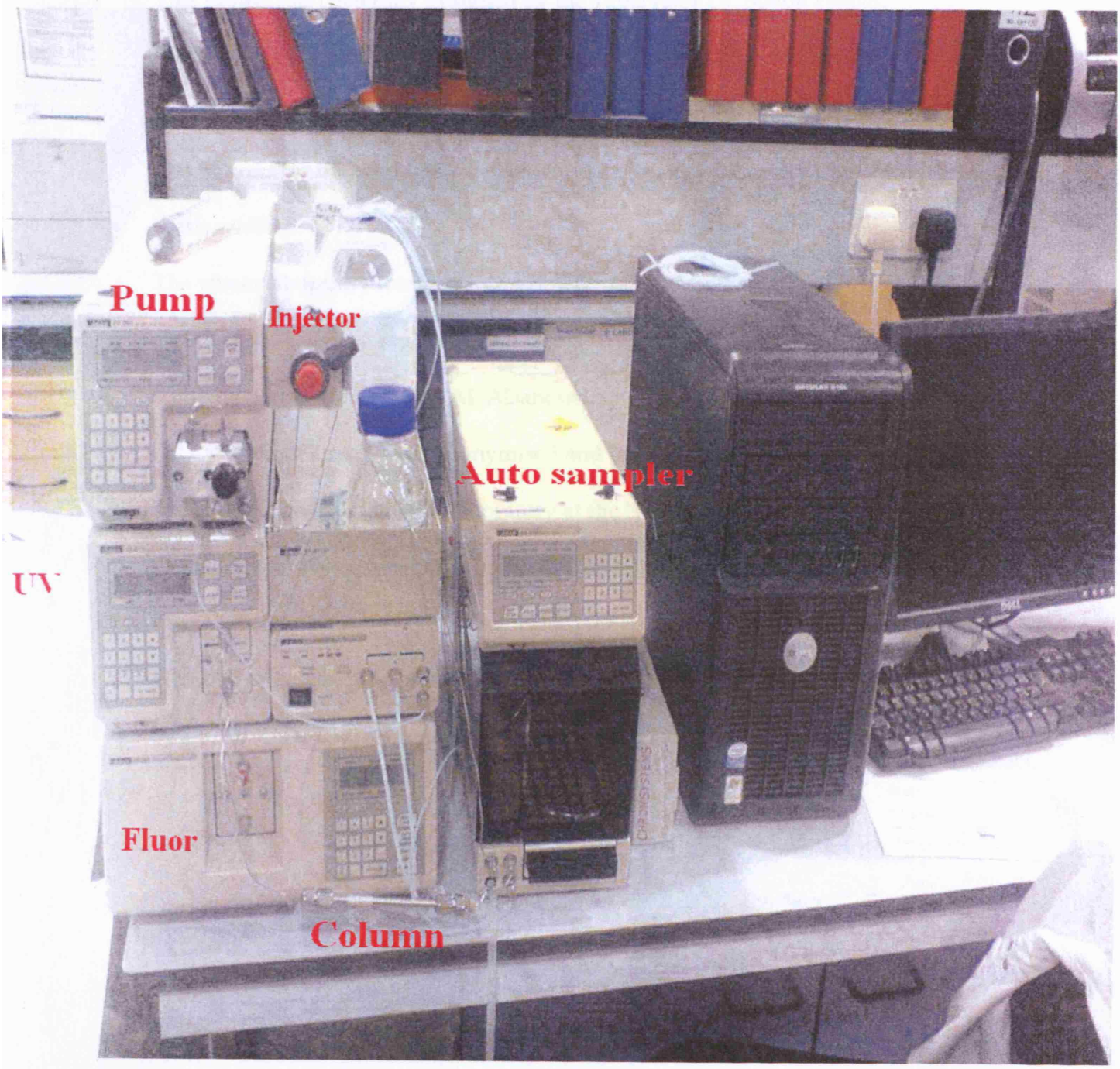
Appendix 2

A photo from the Neurometabolics Lab for

HPLC SYSTEM USED IN THIS STUDY



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Neurometabolic Unit

Statement of Ownership

This project has been fully designed by the supervisor Dr. Simon Heales, The project design include the student training on the HPLC Method, the sample size determination, and the study duration beside full supervision and witness for the whole laboratory work and results documentation and thesis writing.

The whole laboratory work and experiments on HPLC, the data collection and data analysis and reporting and thesis writing were done by the MSc student (clinical Neurology) Ali Alsanousi.

All CSF samples used were anonymised and only coded and stored in the -80°C in the Neurometabolic laboratory at the National Hospital for Neurology and Neurosurgery, Queen square, London -UK.

Two CSF samples were (also anonymised) for ADSL deficiency confirmed patients, these samples (also anonymised) were gifted from collaborating lab by Dr. Keith Hyland (Horizon Molecular Medicine, Atlanta, GA, USA).

UCL-Institute of Neurology
National Hospital for Neurology and Neurosurgery
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