ERNDiM

Quality Assurance in Laboratory Testing for IEM

Title

CSF Neurotransmitters Workshop

Name

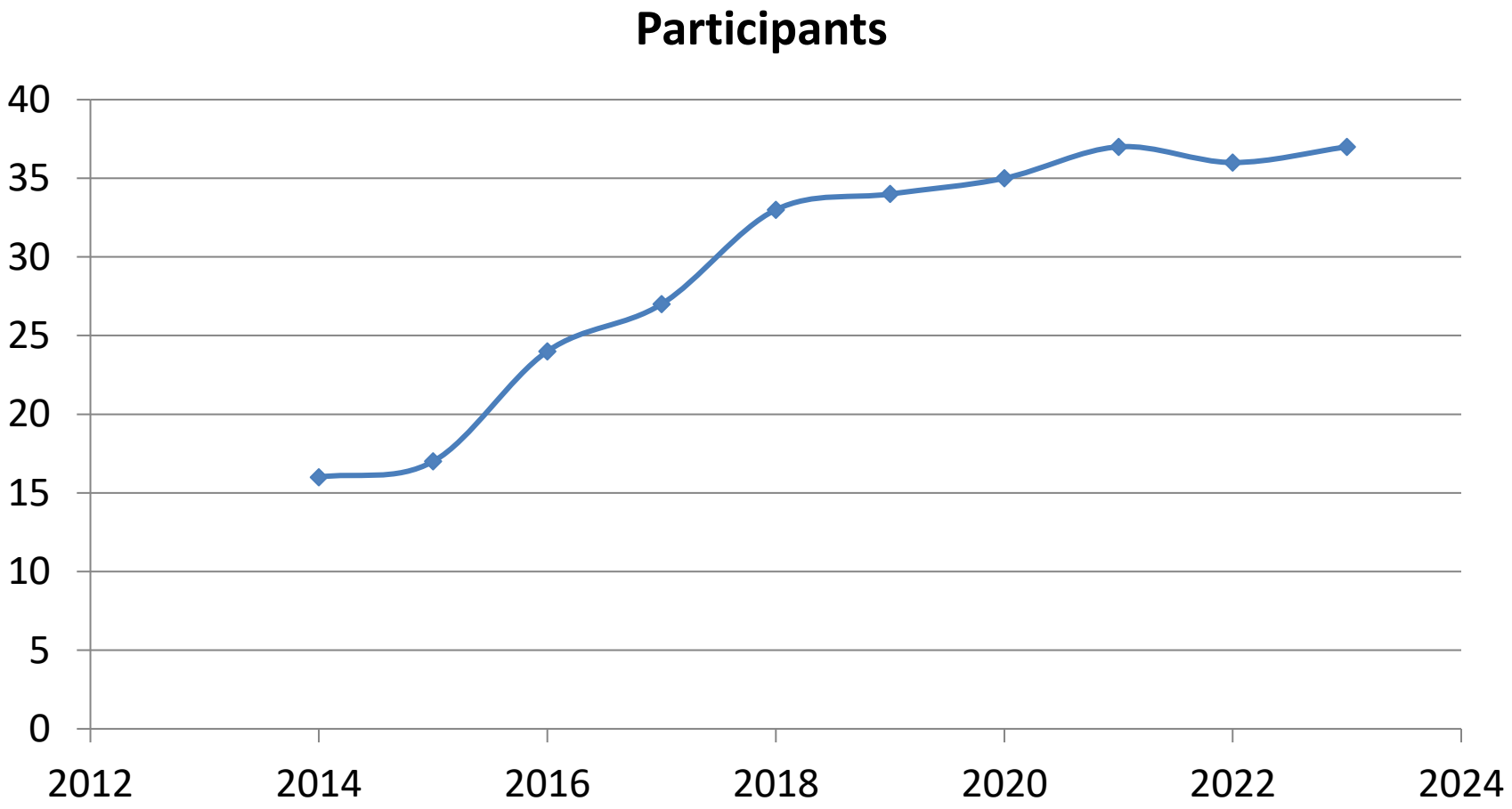
Date

Simon Pope and Rafael Artuch, October 2024

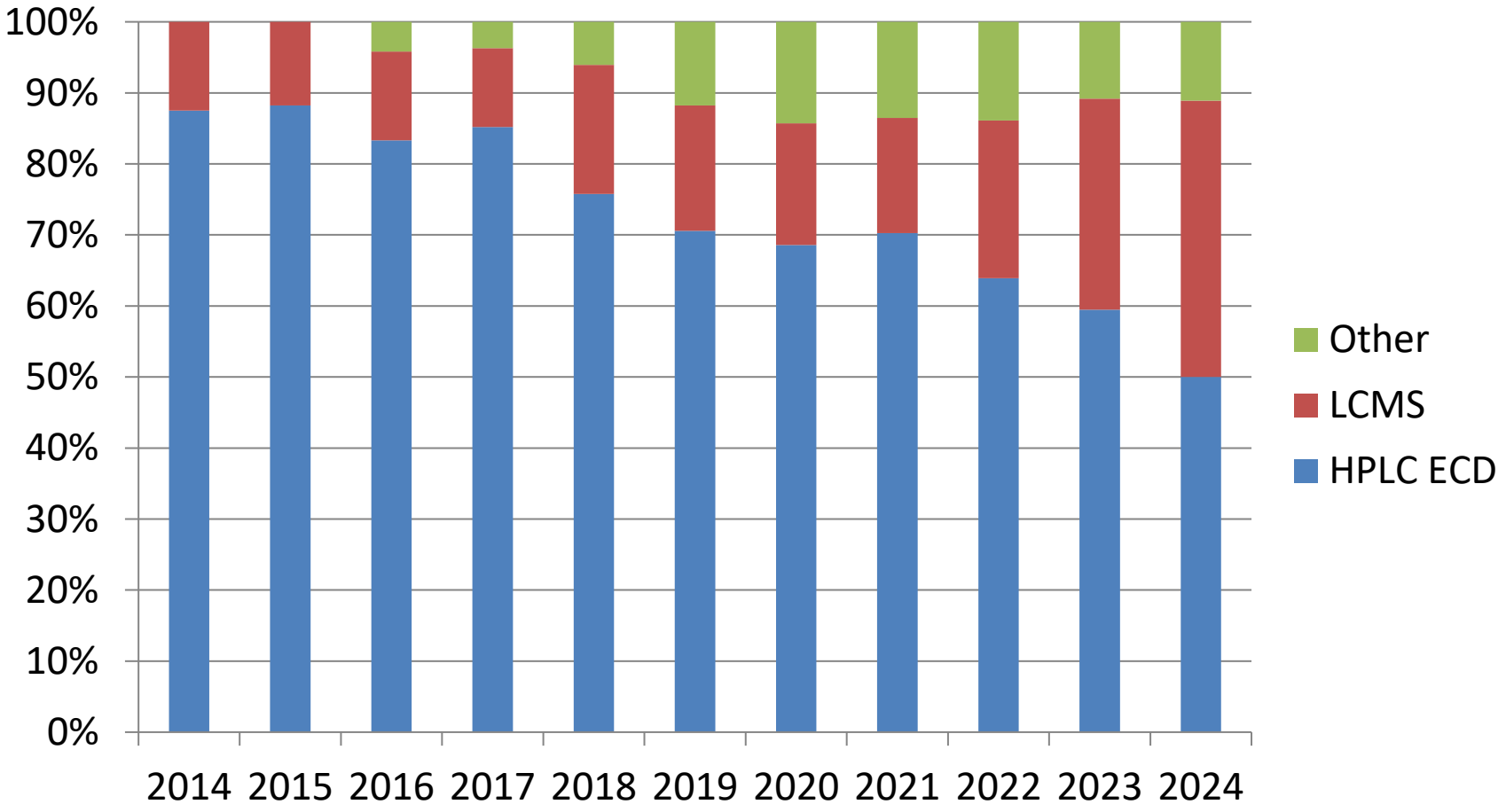
Background and overview

- The ERNDIM CSF neurotransmitter scheme started in 2014
- Interpretation has always been part of the scheme
- 5-methyltetrahydrofolate was added in 2024
- Biopterin and neopterin will be added next year

Participants each year



Methods – change from HPLC ECD to LCMS

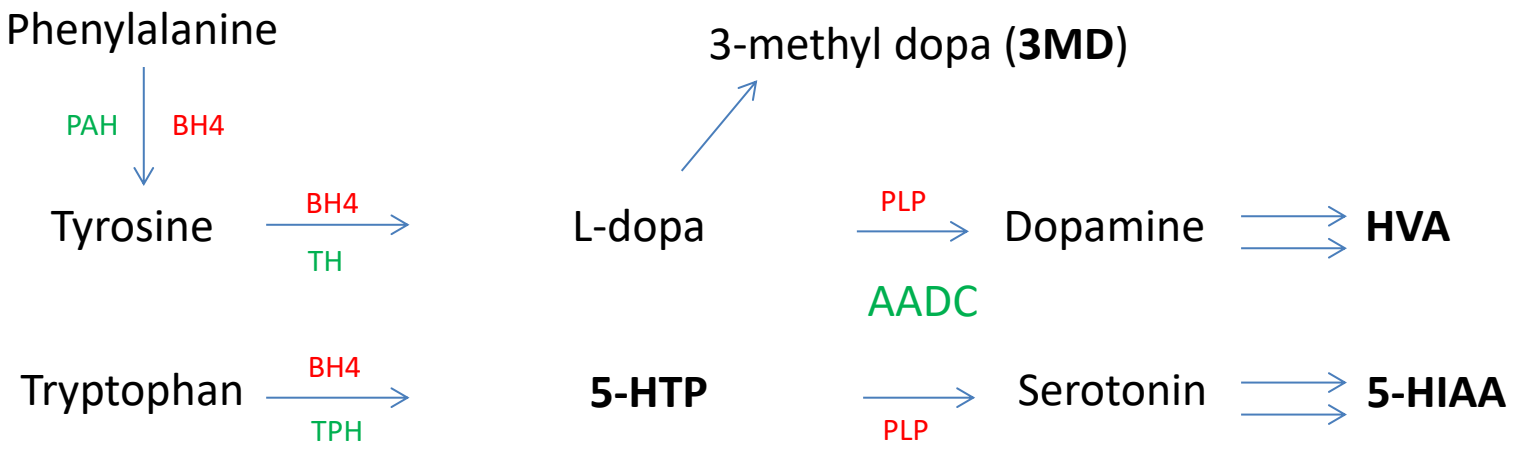


Overview of workshop

- Technical aspects of HPLC and LCMS
  - Monoamines
  - Folate
  - Pterins

  Main focus will be on underivatised methods but some derivatisation methods will be discussed

Dopamine and serotonin metabolism



- HVA is measured as a marker of dopamine turnover
- 5HIAA is a marker of serotonin turnover
- BH4 and PLP are co-factors in dopamine and serotonin synthesis
- 5-methyltetrahydrolate (5MTHF) is used in methylation of L-dopa

Technical aspects – pre-analytical

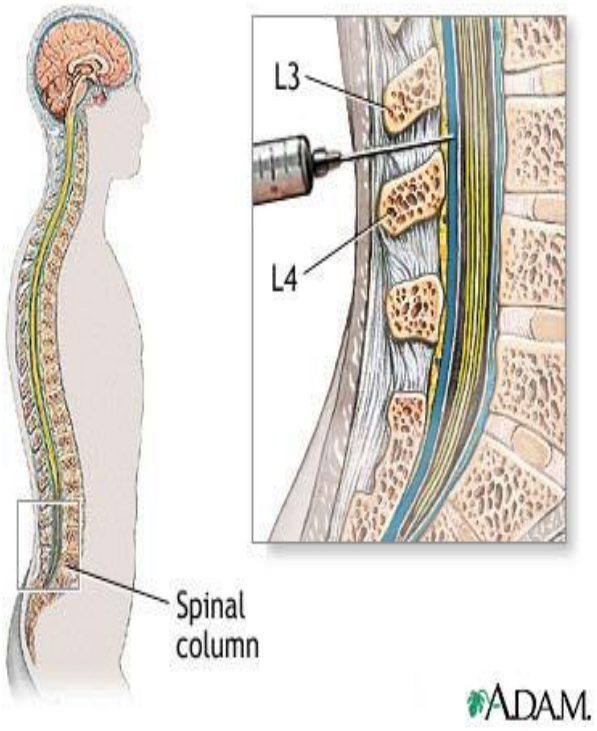
- CSF collection
- Rostrocaudal gradient for 5HIAA, HVA and BH4
- Important to collect specific fractions and have fraction specific reference ranges.
- Some metabolites (BH4, 5HIAA and 5MTHF) are more labile so important to freeze CSF as soon as possible and store at -70C.
- Reducing agents (DTE) and metal chelators can prevent oxidation of BH4

CSF - Sample Requirements

- *Tube 1* 0.5ml **HVA & 5-HIAA**
- *Tube 2* 0.5ml **5-MTHF & PLP**
- *Tube 3* 1.0ml **Pterins**

(DTE/DETAPAC)

Rostro-caudal Gradient



Collect at bedside and freeze immediately
Age related reference ranges
Clinical Details and Drugs

ERNDIM
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Technical aspects – pre-analytical

- Reference ranges are age-related and fraction specific
- Vast differences in concentration of monoamines between lumbar and ventricular samples
- In case of doubt, confirm with clinician

Age-related reference ranges

HVA (nmol/L)	
0 - 4 months	324-1098
4m - 8 months	362-955
8m - 1 year	176-851
1y - 5 years	154-867
5y - Adult	71-565
5HIAA	
0 - 4 months	199-608
4m - 8 months	63-503
8m - 1 year	68-451
1y - 5 years	89-367
5y - Adult	58-220

5-methylterahydrofolate
Reference ranges

Age (years)	CSF reference range (nmol/L)
0-2	72-305
2-5	52-178
5-10	72-172
>10	46-160

Continuous reference ranges

JOURNAL ARTICLE

Cerebrospinal Fluid Homovanillic and 5-Hydroxyindoleacetic Acids in a Large Pediatric Population; Establishment of Reference Intervals and Impact of Disease and Medication

Get access

Helena Rodriguez-Gonzalez, Aida Ormazabal, Mercedes Casado, Angela Y Arias, Clara Oliva, Maria Barranco-Altirriba, Ricard Casadevall, Francesc García-Cuyas, Andrés Nascimento, Carlos Ortez ... Show more

Clinical Chemistry, hvae139, https://doi.org/10.1093/clinchem/hvae139
Published: 27 September 2024 **Article history**

Cite Permissions Share

Abstract

Background

Cerebrospinal fluid (CSF) homovanillic (HVA), and 5-hydroxyindoleacetic acids (5-HIAA) are biomarkers of neurological diseases affecting the dopaminergic and serotoninergic pathways. Establishing reference intervals for these metabolites faces the challenges of a lack of healthy controls and a negative correlation with age, making stratified intervals unrealistic. We propose a pipeline to determine continuous reference intervals for HVA and 5-HIAA using an indirect method. We also studied the confounding effects of different variables and explored the impact of antiepileptic and neuroleptic treatments on HVA and 5-HIAA values.

Methods

The study used least squares regression to fit age–concentration curves from a cohort of pediatric patients (n = 1533), where the residuals represent metabolite values excluding age effect. Presuming that HVA and 5-HIAA primary deficiencies characterize a distinct subpopulation, we fitted a two-component finite mixture model in age-normalized data and set reference intervals at the central 95% of the nondeficient population.

Results

Patients with primary genetic deficiencies of HVA and/or 5-HIAA consistently fall outside the proposed

Sample treatment overview

- CSF is a fairly clean matrix with low protein
- Many ‘traditional’ methods inject neat CSF
- This can lead to column/tubing blockages
- Sample treatment can include centrifugation, molecular weight cut-off filters, protein precipitation (acid or organic)

Analytical methods

- HPLC with ECD for monoamines
- HPLC with fluorescence 5MTHF
- HPLC with ECD and fluorescence pterins
- Mass spectrometry methods

Monoamines by HPLC with ECD

- Phosphate buffer with ion pair (octanesulphonic acid), pH2.7, 18% methanol, C18 column 1.3ml/min
- 50ul injected of filtered CSF. Pall 10kDa filters used. Check filters as some contain preservatives which can affect recovery/ ion pair
- Separation in 20-30 mins
- 5HIAA least stable metabolite but all typically stable for 24 hours at 4C.
- At acidic pH, MHPG and L-dopa run very early and are hard to separate/quantify.

Monoamines by ECD – ECD set up

Original method with recirculating mobile phase using Coulochem electrochemical detector

5010
E1 and E2 coulometric
E1 = 20-50mV
E2 = 400-500mV

Types of electrochemical detector
Coulometric – total oxidation

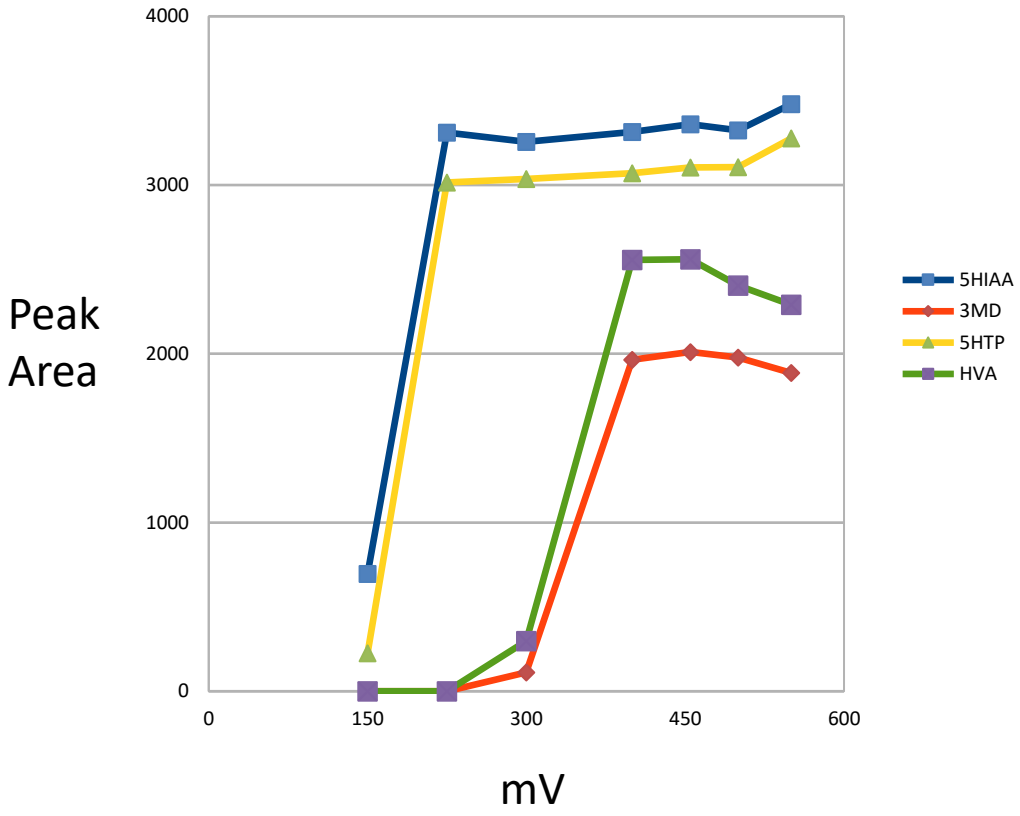
Amperometric – partial oxidation 10%

Adapted method when 5010 no longer made – still able to recirculate

Guard/conditioning cell
Single electrode 20-50mV

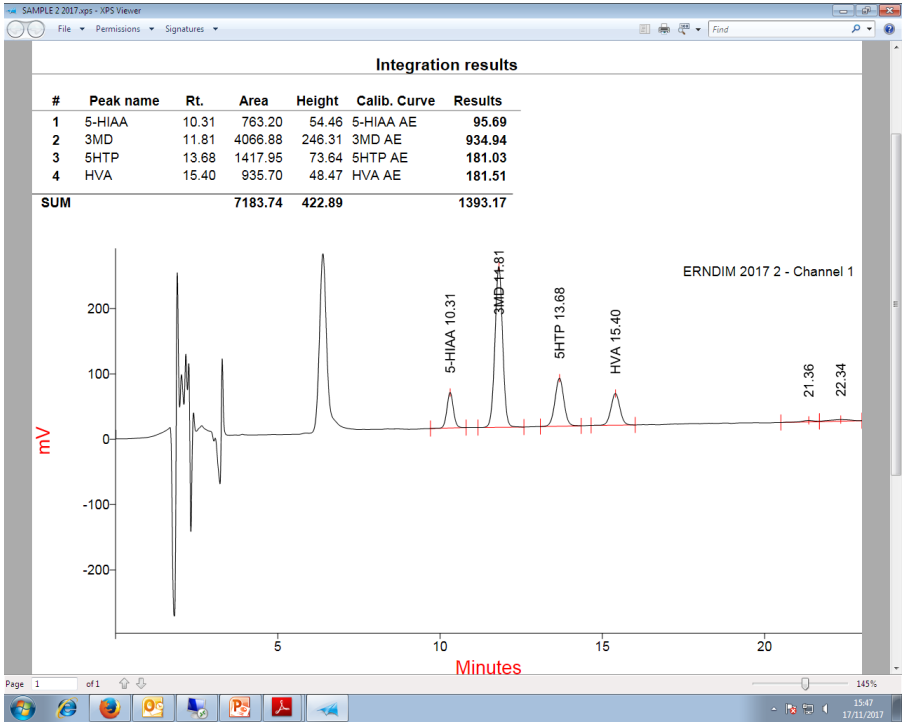
5011
E1 coulometric =400-500mV
E2 amperometric = 0mV

Voltammagram



Very important to check voltammagram and peak areas regularly. 3-methyl dopa hardest to oxidise so changes to voltammagram most likely to affect 3-methyl dopa first. This may explain greater variation for 3-methyl dopa.

Effect of pH – pH2.7



3MD and 5HTP if pH increased

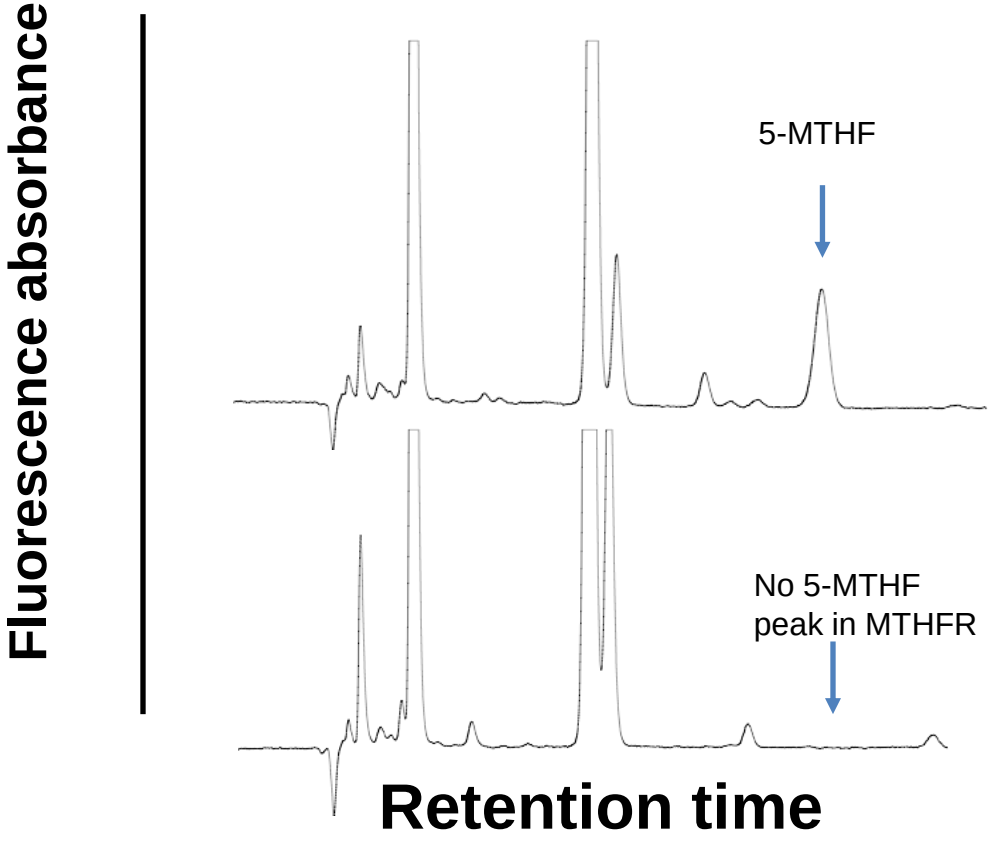
3MD and 5HTP if pH lowered

5-MTHF by HPLC with fluorescence

- Acetate buffer, pH 4.7, 13.5% methanol, recirculated
- Unfiltered CSF unless bloodstained
- 20 minute run time
- Ex 290 Em 358
- Ascorbate/DTE in standard but 5-methyltetrahydrofolate stable in CSF

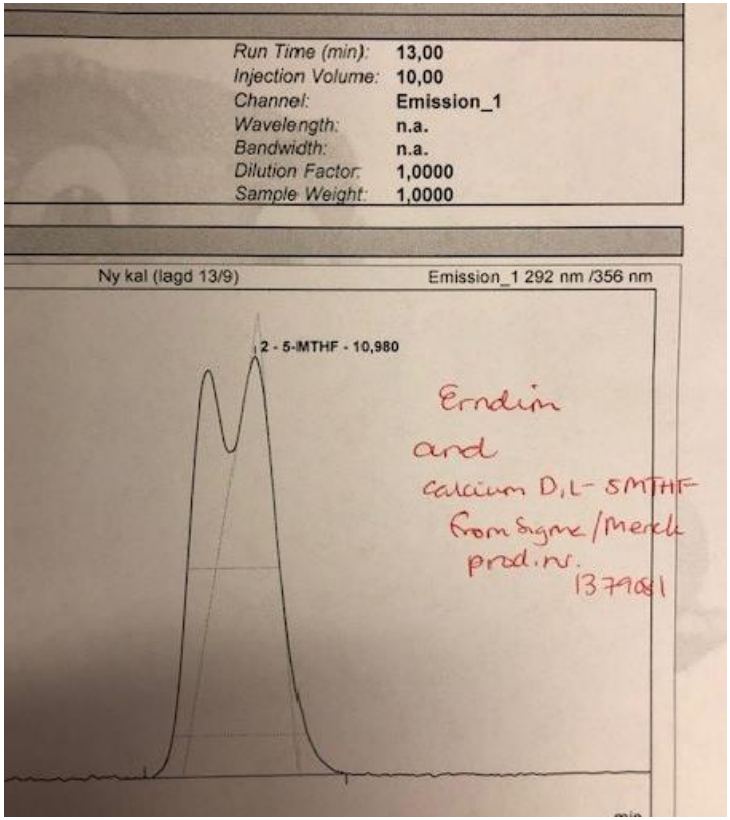
5-methyltetrahydrofolate by HPLC with fluorescence

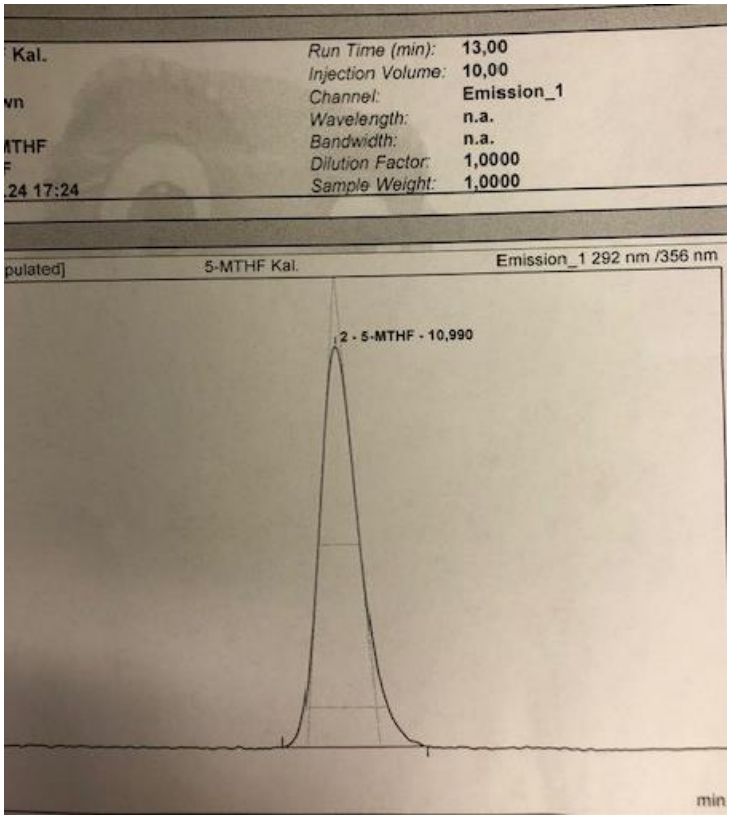
HPLC chromatogram showing absence of 5MTHF peak in CSF of patient with severe MTHFR deficiency



Other fluorescent analytes also seen on chromatogram e.g. tryptophan, 5HIAA, 5HTP

Peak splitting on folate





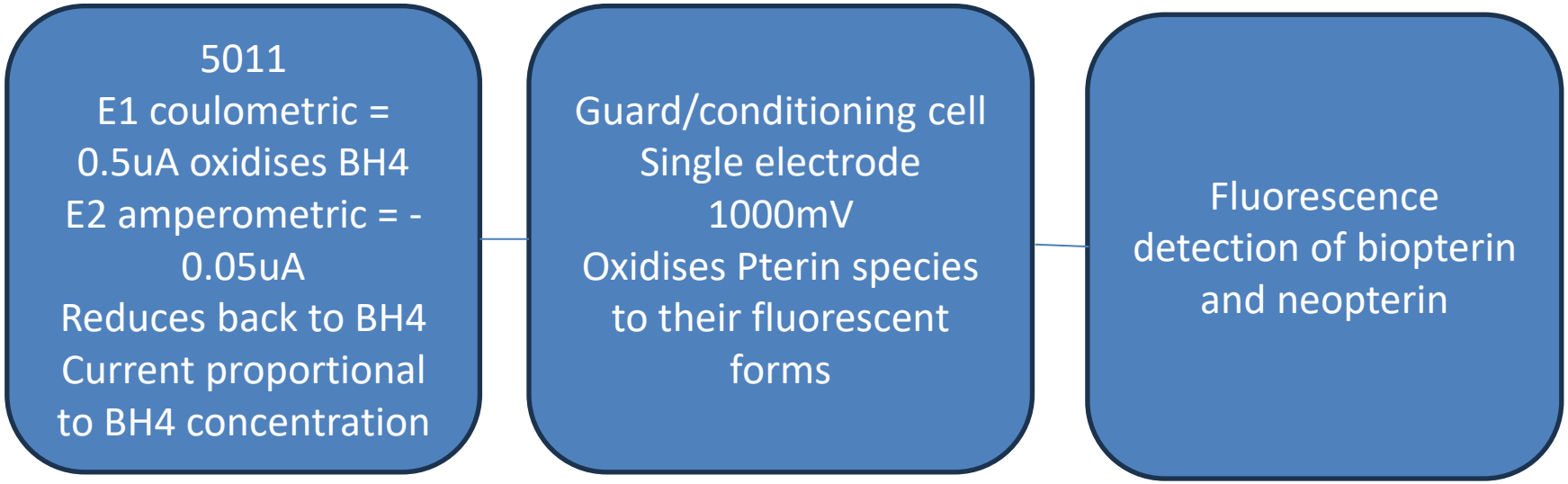
Do other labs see this? Is it due to low pH (2.7)/ion pairing/diastereoisomers?

Pterins by HPLC with ECD and fluorescence

- Acetate Citric acid aqueous buffer with EDTA and DTE – non-recirculating
- C18 column, 1.3ml/min
- Unfiltered CSF with DTE/DETAPAC. Filter if bloodstained. 20 min run time
- BH4 very unstable without DTE/DETAPAC. In biological samples it does not degrade fully to biopterin.
- 30nM BH4 will not degrade to 30nM biopterin. Therefore, important to stabilise BH4 even if using an oxidation method.

Pterins by HPLC with ECD and fluorescence

- Electrochemical/fluorescence set-up



Overview of HPLC with ECD/fluorescence

HPLC ECD/fluorescence
Advantages:
Simple prep
Robust and reproducible
Years of experience
Low nanomolar sensitivity

Disadvantages:
Co-elution
Some electrochemical consumables no longer being manufactured
Not all compounds are electrochemically active/fluorescent

Monoamines and 5MTHF by mass spectrometry - underivatised

LC-MS without derivatisation

- 80µl filtered CSF + 20µl internal standard mix
- 10µl injected
- Separation in 10-15 minutes

- Acquity UPLC HSS T3 1.8µM, 2.1x150mm
- A: Water 0.1% formic acid, B: ACN 0.1% formic acid
- Gradient from 97% aqueous to 95% ACN

Mass spectrometer Waters TQ-XS – higher sensitivity triple quadrupole

All analytes ionised in positive mode except HVA which has better peak-noise in –ve MHPG has low sensitivity. Additives such as ammonium salts have not helped.

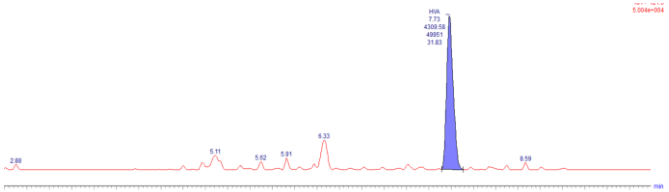
Underivatised LCMS MRMs

Compound	Transition	
3MD (QUAN)	212.053-149.047	
3MD (1)	212.053-195.057	
3MD (2)	212.053-93.007	
5HTP (QUAN)	221.053-204.069	
5HTP (1)	221.053-162.041	
5HTP (2)	221.053-134	
5HIAA (QUAN)	192-145.9	
5HIAA (1)	192-91	
5HIAA (2)	192-118.2	
HVA (QUAN)	181-121.8	
HVA (1)	181-76.9	
MTHF (QUAN)	460.2-313.1	
MTHF (1)	460.2-194.1	
MTHF (2)	460.2-180.1	

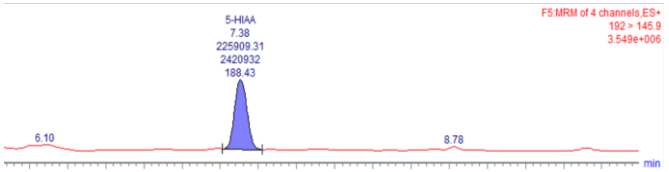
Important to have a stable isotope internal standard for each analyte

Also important to have two transitions for each analyte in case of interfering compounds

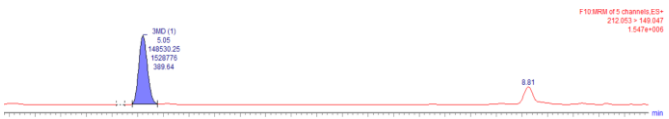
Monoamines and 5MTHF by mass spectrometry - underivatised



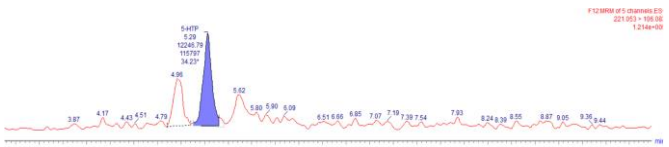
61nM HVA



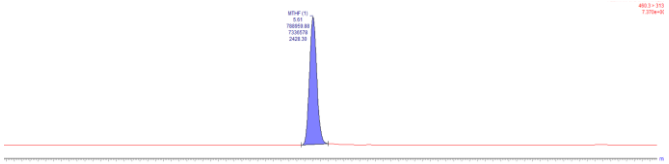
15nM 5HIAA



14nM 3MD

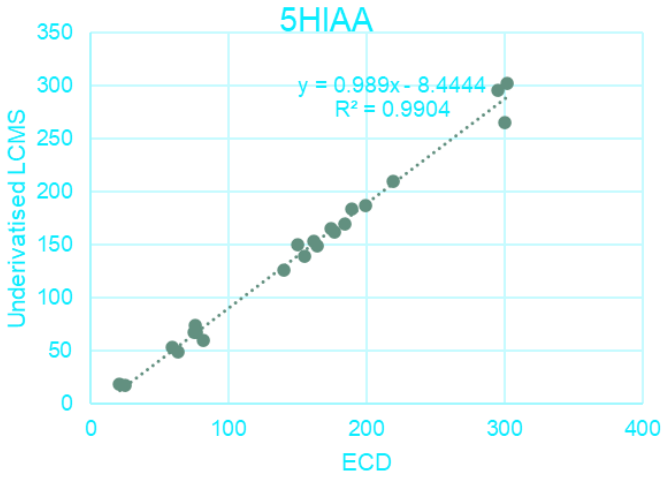


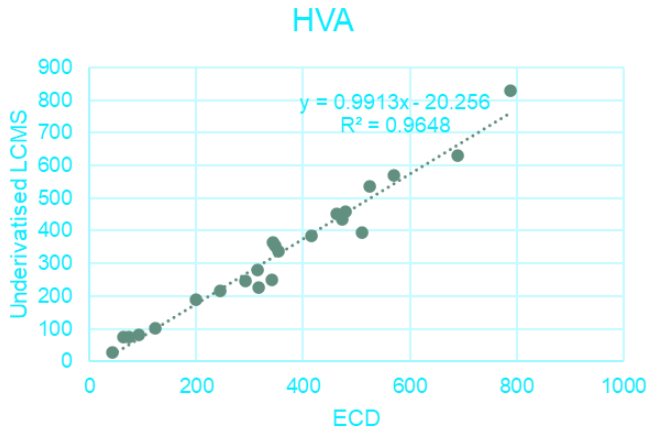
7nM 5HTP



48nM 5MTHF

Good agreement with ECD





Pros and cons of underivatised LCMS method

LC-MS without derivatisation
Advantages:
- Simple prep and short run times
- Low nanomolar sensitivity, good correlation with HPLC ECD
- Many other neurochemicals – e.g. folate, neopterin, amino acids, co-factors can be measured in same run.

Disadvantages:
- Less experience if problems
- Some compounds do not ionise well. HVA is an example.
- However, modification of the gradient and column to improve retention enabled detection at low nanamolar levels, well below bottom of reference range (>76nM).
- Higher sensitivity mass spectrometer required

Derivatisation and LCMS – better sensitivity

LC-MS with benzoyl chloride derivatisation

- 25µl of filtered CSF + 25µl internal standard +
- 25µl Sodium carbonate + 25µl 2% benzoyl chloride in acetonitrile. Derivatisation takes a few minutes.
- 5µl injected.
- Separation in 5-10 minutes

- Acquity UPLC HSS PFP 1.8µM, 2.1 x 100mm
- A: Water, ammonium formate, formic acid, B: ACN, 0.1% formic acid

Derivatisation with benzoyl chloride

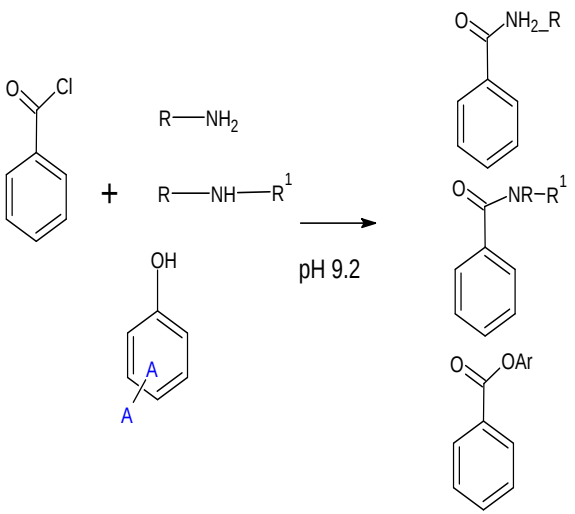
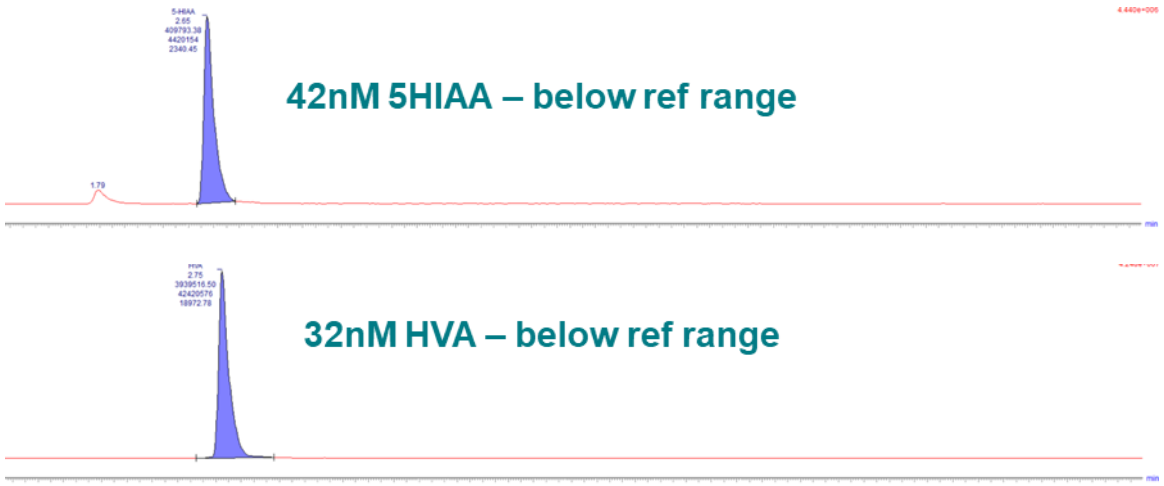


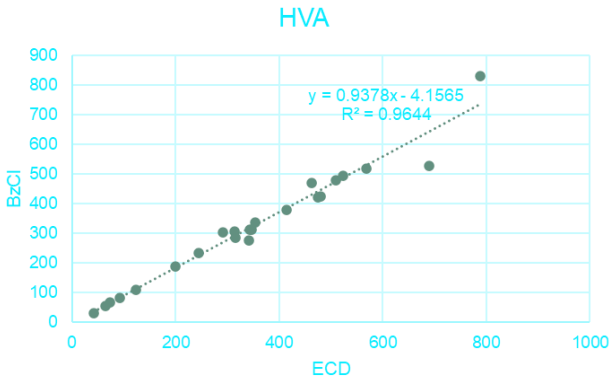
Figure. Benzoyl chloride derivatisation of primary and secondary amines and phenols

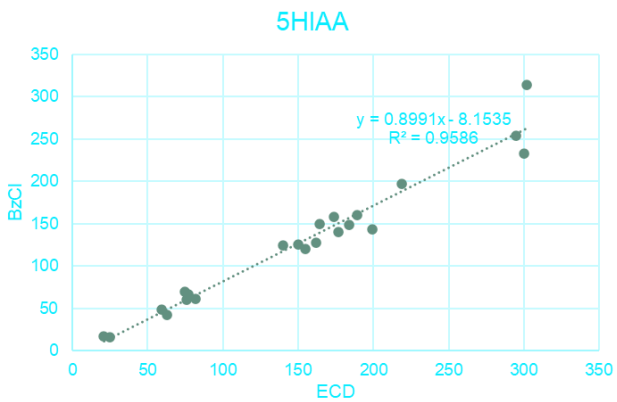
Compound	Adduct detected	Precursor ion (m/z)	Product ion (m/z)	Time (s)	CV (V)	CE (eV)	Internal Standard
MHPG	MHPG-Bz-H	306	105	0.032	20	10	MHPG-D3
HIAA	HIAA-Bz-NH4	313	105	0.032	25	15	HIAA-D5
HVA	HVA-Bz-NH4	304	105	0.032	25	10	HVA-D3
5OHTrp	5OHTrp-H	426	105	0.032	36	40	5OHTrp-D4
3OMD	3OMD-H	420	105	0.032	25	15	3OMD-D5
MHPG-D3	MHPG-D3-Bz-H	309	105	0.032	20	10	
HIAA-D5	HIAA-D5-Bz-NH4	318	105	0.032	25	15	
HVA-D3	HVA-D3-Bz-NH4	307	105	0.032	25	10	
5OHTrp-D4	5OHTrp-D4-H	433	105	0.032	36	40	
3OMD-D5	3OMD-D5-H	425	105	0.032	25	15	

Derivatisation and LCMS



Good agreement with HPLC with ECD





Pros and cons of derivatised LCMS method

LC-MS with benzoyl chloride derivatisation
Advantages:
- Simple prep and short run times
- Low nanomolar sensitivity, good correlation with HPLC ECD
- Derivatisation helps retention on column and ionisation of polar compounds (eg HVA, MHPG)
- >70 neurochemicals have been measured using this derivatisation.

Disadvantages:
- Less experience if problems
- Derivatisation affects fragmentation so less specific' fragments for identification
- Toxic chemicals

General troubleshooting

- Keep good records!
  Pressure, reagents, peak areas, retention times

- Keep standards as stock solutions in aliquots in -70C freezer and monitor performance over time

- Dirty samples are a big problem. Filter or precipitate if possible. Modern UPLC systems and mass spectrometers are very sensitive to particulate

- Make sure needle wash is suitable. Too organic and it can cause precipitation

- Reversing or backflushing HPLC columns can help with poor chromatography

- Adjusting the organic concentration or pH can help to separate peaks

- Periodically run a voltammogram or check MRMs (mass drift can occur)

Useful references – mass spectrometry

Journal of Chromatography A
Volume 1635, 4 January 2021, 461775

Simultaneous determination of 30 neurologically and metabolically important molecules: A sensitive and selective way to measure tyrosine and tryptophan pathway metabolites and other biomarkers in human serum and cerebrospinal fluid

Zsolt Galla [a], Cecília Rajda [b], Gábor Rácz [a], Nóra Grecsó [a], Ákos Baráth [a], László Vécsei [b c], Csaba Bereczki [a], Péter Monostori [a]

Biomedical Chromatography

RESEARCH ARTICLE

Quantification of monoamine biomarkers in cerebrospinal fluid: Comparison of a UHPLC–MS/MS method with a UHPLC coupled to fluorescence detection method

Ayoub Boulghobra, Taous Abar, Fathi Moussa, Bruno Baudin, Diana Rodriguez, Antoine Pallandre, Myriam Bonose

First published: 09 September 2022 | https://doi.org/10.1002/bmc.5502 | Citations: 1

Funding information: Agence Nationale de la Recherche, Grant/Award Number: 18-CE09-0042; Ile de France region, Grant/Award Number: SYNALGIA

J Chromatogr A. Author manuscript; available in PMC 2017 May 13.
Published in final edited form as:
J Chromatogr A. 2016 May 13; 1446: 78–90.
Published online 2016 Apr 4. doi: 10.1016/j.chroma.2016.04.006

PMCID: PMC4845038
NIHMSID: NIHMS777689
PMID: 27083258

Author Manuscript

Benzoyl Chloride Derivatization with Liquid Chromatography-Mass Spectrometry for Targeted Metabolomics of Neurochemicals in Biological Samples

Jenny-Marie T Wong,[1,*] Paige A Malec,[1,*] Omar S Mabrouk,[1,2] Jennifer Ro,[3] Monica Dus,[4] and Robert T Kennedy[1,2]

Anal Chem. 2020 Jul 7; 92(13): 9072–9078.
Published online 2020 Jun 2. doi: 10.1021/acs.analchem.0c01263

PMCID: PMC7349590
PMID: 32484659

Journal Article

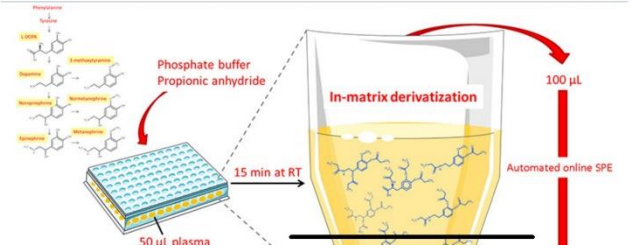
In Matrix Derivatization Combined with LC-MS/MS Results in Ultrasensitive Quantification of Plasma Free Metanephrines and Catecholamines

Martijn van Faassen,[†] Rainer Bischoff,[‡] Karin Eijkelenkamp,[§] Wilhelmina H. A. de Jong,[†∥] Claude P. van der Ley,[†] and Ido P. Kema[*†]

Associated Data

▸ Supplementary Materials

Abstract Go to: ▸



Useful references – reference ranges, folates and pterins

Molecular Genetics and Metabolism
Volume 95, Issue 3, November 2008, Pages 127-132

Technical and biochemical factors affecting cerebrospinal fluid 5-MTHF, biopterin and neopterin concentrations

M.M. Verbeek [a b], A.M. Blom [a c], R.A. Wevers [a b], A.J. Lagerwerf [a], J. van de Geer [a], M.A.A.P. Willemsen [c]

LC-MS/MS Analysis of Cerebrospinal Fluid Metabolites in the Pterin Biosynthetic Pathway.

Arning E [1], Bottiglieri T [1]

Author information

JIMD Reports, 12 Sep 2014, 29:1-9
https://doi.org/10.1007/8904_2014_336 PMID: 25213568 PMCID: PMC5059177

JIMD JOURNAL OF INHERITED METABOLIC DISEASE SSIEM

REVIEW

Cerebral folate deficiency: Analytical tests and differential diagnosis

Simon Pope, Rafael Artuch, Simon Heales, Shamima Rahman

First published: 27 March 2019 | https://doi.org/10.1002/jimd.12092 | Citations: 60

9:01 PM Thu 10 Oct nature.com

Regular Article | Published: 01 July 1993

Cerebrospinal Fluid Concentrations of Pterins and Metabolites of Serotonin and Dopamine in a Pediatric Reference Population

Keith Hyland, Robert A H Surtees, Simon J R Heales, Ann Bowron, David W Howells & Isabel Smith

Pediatric Research **34**, 10–14 (1993) | Cite this article

2030 Accesses | **114** Citations | Metrics

Abstract

ABSTRACT: Accurate diagnosis and management of inborn errors of monoamine neurotransmitter and tetrahydrobiopterin metabolism depend on reliable reference ranges of key metabolites. Cerebrospinal fluid (CSF) was collected in a standardized way from 73 children and young adults with neurologic disease, with strict exclusions. In each specimen, concentrations of homovanillic acid (HVA), 5-hydroxyindoleacetic acid (HIAA), total neopterin, 7,8-dihydrobiopterin, and tetrahydrobiopterin (BH4) were measured using HPLC. There was a continuous decrement in CSF HVA, HIAA, and BH4 during the first few years of life; this was independent of height (or length). Age-related reference ranges for each metabolite are given. Extensive correlations between HVA, HIAA, 7,8-dihydrobiopterin, and BH4 were further analyzed by multiple regression. Age and CSF BH4 were significant explanatory variables for CSF HIAA, but CSF HVA had only HIAA as a significant explanatory variable.

Questions and discussion

- 5MTHF analysis in CSF: tips and tricks
- Double peak for the 5MTHF in the Erndim sample. If it`s possible, if someone will share their LCMSMS method. Thank you .
- Have any LC-MS peers encountered problems with HVA precision? We run it in positive mode and the ionization is not quite stable.
- HPLC with Electrochemical Detection, Coulochem III. Advantages and troubleshooting.
- Possibility of adding HMPG and pterins in future schemes
- Pre analytical aspects for CSF pterin analysis. Tips for method transfer of biogenic amines and pterins from HPLC to TMS