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## ► To cite this version:

Elodie Lamy, Léa Pilyser, Claire Paquet, Elodie Bouaziz-Amar, Stanislas Grassin-Delyle. High-sensitivity quantification of acetylcholine and choline in human cerebrospinal fluid with a validated LC-MS/MS method. *Talanta*, 2021, 224, 10.1016/j.talanta.2020.121881 . hal-03188148

**HAL Id: hal-03188148**

**<https://hal.science/hal-03188148>**

Submitted on 2 Jan 2023

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# **High-sensitivity quantification of acetylcholine and choline in human cerebrospinal fluid with a validated LC-MS/MS method**

Running head: acetylcholine and choline in human CSF

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27 **ABSTRACT**

28 Acetylcholine is the neurotransmitter of the parasympathetic nervous system, synthesized from  
29 choline and involved in several neurodegenerative diseases. Exploration of cholinergic  
30 neurotransmission in the human central nervous system is limited by the lack of a sensitive and  
31 specific method for the determination of acetylcholine and choline expression. We developed an  
32 hydrophilic interaction liquid chromatography – mass spectrometry method for the quantification of  
33 both molecules in human cerebrospinal fluid samples. An extensive selectivity study towards  
34 endogenous interfering compounds, in particular  $\gamma$ -butyrobetain, was performed and the method  
35 was validated according to the European Medicine Agency and Food and Drug Administration  
36 guidelines for the validation of bioanalytical methods. The performance of the method was  
37 excellent with a lower limit of quantification at 5 ng/L (34.2 pmol/L) for acetylcholine and 5  $\mu$ g/L  
38 for choline, a precision in the range 1.3-11.9% and an accuracy between 85.2 and 113.1%. This  
39 suitability of the method for the quantification of acetylcholine and choline in clinical samples was  
40 demonstrated with the analysis of patient cerebrospinal fluid samples. Altogether, this validated  
41 method allows the simultaneous quantitative analysis of acetylcholine and choline in human  
42 cerebrospinal fluid with high sensitivity and selectivity. It will allow to better characterize the  
43 cholinergic neurotransmission in human pathologies and to study the effects of drugs acting on this  
44 system.

45

46 **Keywords:** acetylcholine; choline; human cerebrospinal fluid; liquid chromatography; mass  
47 spectrometry.

48

49 **INTRODUCTION**

50 Acetylcholine (ACh) is the mediator of the parasympathetic nervous system, identified by Dale and  
51 Dudley in 1929 [1] and playing roles in neuromuscular junctions in the periphery and in memory  
52 and learning in the central nervous system. ACh is synthesized from acetyl-CoA and choline (Ch),  
53 then released in the synaptic cleft by presynaptic neurons where it acts on muscarinic and nicotinic  
54 receptors and is at last subjected to rapid metabolism under the action of acetylcholinesterase and/or  
55 butyrylcholinesterase enzymes, resulting in the formation of choline and acetate. A deficiency of  
56 cholinergic neurotransmission is observed in conditions such as Alzheimer disease (AD) [2] and  
57 drug therapy with acetylcholinesterase inhibitors is expected to increase brain ACh concentrations  
58 and is used for long as a pharmacological strategy [3].

59 To date, all reported human ACh cerebrospinal fluid (CSF) concentrations were measured with  
60 colorimetry [4], radioimmunoassay [5], gas chromatography-mass spectrometry (GC-MS) [6] or  
61 high performance liquid chromatography with electrochemical detection [7-13] but numerous  
62 methods have been published more recently for the quantification of ACh with liquid-  
63 chromatography – electrospray mass spectrometry (LC-ESI-MS), although all used rat, murine or  
64 bovine tissues [14-23] or human non-CSF samples [24-26]. Such LC-MS methods allowed to reveal  
65 a risk of lack of selectivity, since the endogenous compound  $\gamma$ -butyrobetain (also known as 3-  
66 dehydroxycarnitine or (3-carboxypropyl)trimethylammonium), a precursor of carnitine, shares the  
67 exact same molecular formula as ACh and is therefore susceptible of interfering with the detection  
68 of ACh. Despite some of the previous mass spectrometry methods reported the quantification of  
69 both ACh and Ch [7, 18-20, 23, 24, 26], only few (and none in human CSF) were able to selectively  
70 distinguish ACh from  $\gamma$ -butyrobetain [18, 21, 22, 25]. In addition; none were validated according to  
71 approved guidelines and their lower limit of quantification (LLOQ) for ACh was in the range 0.25-  
72 300 fmol on column [14, 15, 17-22], with even higher LLOQ for GC-MS studies [7, 27, 28].

73 With respect to human CSF ACh concentrations reported to date, the ranges were between  $6.14 \pm$   
74  $1.39$  pmol/mL to  $393.6 \pm 142.5$  pmol/mL in control patients [4, 7, 8, 11, 12],  $0.1$ - $0.6$  pmol/mL to  
75  $10.7 \pm 5.1$  pmol/mL in AD patients [5, 7, 8, 11, 12],  $124.3 \pm 56.4$  pmol/mL in multiple sclerosis  
76 patients [4],  $5$ - $50$  pmol/mL in surgery patients [9],  $11$ - $85$  pmol/mL in women with painful labor or  
77 elective caesarian section [10] and  $1980 \pm 740$  pmol/mL in patients with different neurological  
78 diseases [13] with number of patients below the detection limit [7], *i.e.* a 64- to 107-fold difference  
79 between studies in the same patient populations, with extreme values of  $0.1$  and  $1980$  pmol/mL (4-  
80 log units). This huge heterogeneity strongly highlights the need for highly-selective and fully  
81 validated methods for the quantitation of ACh in human CSF. In the present study, we report a  
82 validated, selective and highly sensitive hydrophilic interaction liquid chromatography tandem mass  
83 spectrometric method (HILIC-MS/MS) and its application for the simultaneous quantification of  
84 acetylcholine and choline in human CSF samples, with an extensive investigation of selectivity  
85 towards  $\gamma$ -butyrobetain.  
86

## 87 MATERIALS AND METHODS

88

### 89 Chemicals and reagents

90 Acetylcholine chloride [2-acetyloxyethyl(trimethyl)azanium;chloride] ( $C_7H_{16}ClNO_2$ ), choline  
91 chloride ( $C_5H_{14}ClNO$ ),  $\gamma$ -butyrobetain chloride [4-(trimethylazaniumyl)butanoate;chloride]  
92 ( $C_7H_{16}ClNO_2$ ) and neostigmine bromide were supplied by Sigma Aldrich (Saint Quentin Fallavier,  
93 France). The internal standard (IS) acetylcholine bromide- $d_{16}$  (ACh- $d_{16}$ ,  $C_7D_{16}BrNO_2$ ) was supplied  
94 by Alsachim (Illkirch, France). LC-MS-grade formic acid was supplied by Sigma Aldrich (Saint  
95 Quentin Fallavier, France). LC-MS-grade methanol, acetonitrile, ammonium formate, acetic acid  
96 and water were from Fisher Scientific (Illkirch, France). Phosphate Buffered Saline (PBS) was  
97 supplied by Eurobio (Les Ulis, France).

98

### 99 Working, calibration standard and quality control solutions

100 Stock solution of ACh, Ch,  $\gamma$ -butyrobetain and IS (1 g/L) were prepared in methanol. Working  
101 solutions for calibration standards (CS) and quality control (QC) were prepared separately by  
102 dilution of each stock solution in methanol. Working solution of IS (1000 ng/L) was obtained by  
103 dilution of the stock solution in methanol. PBS was used as a surrogate matrix to CSF to prepare  
104 calibration standards and quality control samples. Calibration curves were prepared by spiking 50  
105  $\mu$ L of PBS with 5  $\mu$ L of the appropriate above-mentioned working solution in order to produce the  
106 CS equivalent to 5.0, 10.0, 15.0, 25.0, 50.0, 100.0, 150.0 and 200.0 ng/L for ACh and to 5.0, 10.0,  
107 50.0, 100.0, 250.0, 500.0, 750.0 and 1000.0  $\mu$ g/L for Ch. Calibration curves were also constructed  
108 for  $\gamma$ -butyrobetain in the range 1-25  $\mu$ g/L as a part of the extended selectivity study. Quality control  
109 samples were prepared at 3 levels: 12, 40 and 175 ng/L for ACh and 15.0, 300.0 and 800.0  $\mu$ g/L for  
110 Ch. QC samples were added with 1  $\mu$ M of the acetylcholine esterase inhibitor neostigmine in order  
111 to avoid ACh degradation.

**112 Sample, calibration standard and quality control preparation**

113 CSF samples were defrosted slowly in ice (about 1 hour). Fifty microliters of each CS, QC or  
114 sample were put into conic 1.5 mL tubes, 5  $\mu$ L of IS solution (1000 ng/L acetylcholine-d<sub>16</sub> in  
115 methanol) and 50  $\mu$ L of acetonitrile at -20°C were then added. Samples were vortexed and  
116 centrifuged at 17,000 x g for 10 minutes at 4°C. Fifty microliters of the supernatant were taken to  
117 injection vials for LC-MS/MS analysis.

118

**119 LC-MS/MS system and analysis procedure**

120 Method optimization was performed for the analytical conditions such as chromatographic column  
121 and mobile phase composition to setup the most sensitive and fastest method. The final  
122 chromatography and mass spectrometry parameters were as follows. Chromatography was  
123 performed with an UltiMate 3000 Quaternary Rapid Separation Pump (Thermo Scientific Dionex,  
124 Villebon, France), using a 1.7  $\mu$ m Acquity UPLC BEH HILIC column (100 x 2.1 mm i.d.) (Waters,  
125 Guyancourt, France) maintained at 35°C in an UltiMate 3000 Rapid Separation Thermostated  
126 Column Compartment (Thermo Scientific Dionex). Ten microliters (10  $\mu$ L) were injected in the  
127 chromatographic system with an UltiMate 3000 Rapid Separation Autosampler (Thermo Scientific  
128 Dionex) with a sample stack maintained at +8°C. The mobile phase was a gradient of acetonitrile  
129 and a 200 mM ammonium formate buffer adjusted to pH 5.5 using formic acid. The starting  
130 composition was 95% acetonitrile, reaching 88% at 7 min. Then, initial conditions were restored  
131 and maintained for 11 minutes. The flow rate was 500  $\mu$ L/min and total run time was 18.0 min.  
132 Compounds were ionized with a heated electrospray ionization source and detected with a triple  
133 quadrupole Quantiva mass spectrometer (ThermoFisher) using multiple reaction monitoring  
134 (MRM). Nitrogen (N<sub>2</sub>-45 nitrogen generator, VWR International, Fontenay sous bois, France) was  
135 employed as sheath and auxiliary gas and argon (Messer, Puteaux, France) was used as a collision  
136 gas. The electrospray source was set in positive ionization. Instrument parameters were optimized

137 using a continuous 20  $\mu$ L/min infusion of ACh, Ch,  $\gamma$ -butyrobetain and internal standard (1 mg/L in  
138 mobile phase). The source parameters were as follows: ion spray voltage: 5200V; vaporizer  
139 temperature: 100°C; sheath and aux gas pressure: 40 and 15 arbitrary units, respectively; ion  
140 transfer tube temperature: 350°C; collision gas pressure: 1.5 mTor; Q1 and Q3 resolution: 0.7  
141 FWHM, RF lens: 39V and cycle time: 1s. The transitions selected for the analysis of each  
142 compound were as follows (precursor ion ( $m/z$ )  $\rightarrow$  product ions ( $m/z$ ) (collision energy (V))): 146.1  
143  $\rightarrow$  87.1 (13), 146.1  $\rightarrow$  43.2 (26) for Ach and  $\gamma$ -butyrobetain, 104.4  $\rightarrow$  60.2 (16), 104.4  $\rightarrow$  45.2 (21)  
144 for Ch and 162.2  $\rightarrow$  94.1 (16), 162.2  $\rightarrow$  46.2 (30) for the internal standard ; the first transition being  
145 used for quantification. Data acquisition and processing was performed using Xcalibur v4.2.28.14  
146 (Thermofisher).

147

#### 148 **Method validation procedure**

149 The developed method was validated according to the European Medicines Agency (EMA)  
150 guideline (EMEA/CHMP/EWP/192217/2009 Rev.1 Corr.2) and Food and Drug Administration  
151 (FDA) guideline (Bioanalytical Method Validation - Guidance for Industry, May 2018) for the  
152 following parameters: selectivity, carry-over, lower limit of quantification, calibration curve,  
153 accuracy, precision, matrix effect and stability.

154

#### 155 *Selectivity and carry over*

156 Six blank samples were processed and compared to samples spiked with the internal standard and  
157 with each compound at the lower limit of quantification. To evaluate the carry-over, a blank sample  
158 was analysed immediately after the highest CS in each run. Assay selectivity and carry-over were  
159 defined by a mean area of less than 20% for each molecule and less than 5% for the IS in blank  
160 samples injected before the lower CS (for selectivity) or just after the highest CS (for carry-over) in  
161 comparison to the signal obtained for the LLOQ.



162 *Lower limit of quantification*

163 The LLOQ was defined as the lowest concentration measurable with a signal superior or equal to 5  
164 times the signal of a blank sample, an accuracy between 80% and 120% and a precision with a  
165 coefficient of variation of  $\pm 20\%$  or less over six measurements. The LLOQ was used as the lowest  
166 calibration standard.

167

168 *Calibration curve*

169 Calibration curves included a blank sample, a zero sample, and eight CS. Quantitation was achieved  
170 by plotting the peak area ratios of each compound to the internal standard versus concentration  
171 followed by regression analysis, excluding blank and zero samples. Back-calculated concentrations  
172 of at least six CS out of the eight (including the LLOQ) had to be within 85-115% of the nominal  
173 concentrations, except for the LLOQ for which the range was 80-120%.

174

175 *Accuracy and precision*

176 Accuracy (measured value/nominal value) and precision (coefficient of variation (CV)) were  
177 determined for the three QC levels and for the LLOQ. Six replicates of each QC level were  
178 processed the same day for the intraday assay, whereas each QC level was processed six times three  
179 different days. To further assess the selectivity and ensure that  $\gamma$ -butyrobetain would not affect the  
180 quantification of ACh and Ch,  $\gamma$ -butyrobetain was also spiked at different concentrations in the QC  
181 samples. The data was analysed using analysis of variance. For QCs, accuracy had to be within 85-  
182 115% of the nominal values and precision of  $\pm 15\%$ , while values between 80-120% and  $\pm 20\%$  were  
183 accepted for accuracy and precision of the LLOQ, respectively.

184

185 *Precipitation recovery, matrix effect and overall recovery*

186 Three procedures (A, B and C) were performed at two QC concentrations (corresponding to the low  
187 and higher QC) to evaluate these parameters: (A) ACh, Ch and IS were spiked in blank PBS and the  
188 complete sample preparation procedure was carried through; (B) blank PBS samples were prepared  
189 using the complete sample preparation procedure, 50  $\mu$ L of supernatant were transferred as usual at  
190 the end of the precipitation step, then ACh, Ch and IS were spiked and the preparation procedure  
191 finished; (C) ACh, Ch and IS were spiked in 100  $\mu$ L of water/acetonitrile (50/50, v/v) and directly  
192 injected.

193 The chromatographic peak areas obtained using the three procedures for the two lowest and highest  
194 QC levels were analysed for each compound and internal standard. Precipitation recovery was  
195 defined by the ratio between the peak area obtained in procedure A and the peak area obtained in  
196 procedure B. Matrix effect was assessed by calculating the matrix factor, defined as the ratio  
197 between the peak area obtained in a blank matrix spiked with ACh, Ch and IS (procedure B) and the  
198 peak area obtained in a pure solution of the analytes in absence of matrix (procedure C). The IS-  
199 normalised matrix factor was calculated by dividing the matrix factor of each compound by the  
200 matrix factor of the IS. The CV of this IS-normalised matrix factor had to be less than 15%. Overall  
201 recovery was defined as the ratio between the peak area obtained in procedure A and the peak area  
202 obtained in procedure C. Overall method recovery of the IS had to be  $\pm 15\%$  of each compound  
203 recovery.

204 In addition to this procedure in the surrogate matrix, matrix effect was also evaluated for the  
205 internal standard ACh-d<sub>16</sub> (100 ng/L) in human CSF samples ( $n = 6$ ).

206

207 *Stability*

208 The stability of processed samples was studied after storage for 24 hours at +4°C. The long-term  
209 stability of QC samples was studied for 3 months at -80°C.

**210 Analysis of study samples**

211 To confirm the suitability for the analysis of clinical samples, the developed method was applied to  
212 measure ACh and Ch concentrations in CSF samples from 10 patients included in the “Cholinergic  
213 Markers as Predictive Tools of Therapeutic Response in Alzheimer's Disease (CHOLINE)” study  
214 (NCT03090854). The study was approved by an ethics committee and all participants gave written  
215 informed consent. Lumbar CSF samples (1-2 mL) were collected according to a standardized  
216 protocol [29]. Neostigmine (200 nM) was added to the samples immediately after sampling.  
217 Samples were then centrifuged (1000 x g at +4°C for 10 min) less than 4 hours after collection,  
218 aliquoted into polypropylene tubes and stored at -80°C in the Lariboisière Hospital biobank (APHP  
219 Université de Paris BB-0033-00064) until analysis.

220

## 221 **RESULTS AND DISCUSSION**

222

### 223 **LC-MS/MS optimization**

#### 224 *Column optimization*

225 ACh, Ch and  $\gamma$ -butyrobetain are hydrophilic compounds (XLogP3 = 0.2, -0.4 and 0.8, respectively),  
226 therefore the performances of different polar analytical columns were compared. The following  
227 columns were tested: Hypercarb (Thermo Scientific) 3.0  $\mu\text{m}$ , 100 x 2.1 mm i.d.; Accucore Urea  
228 HILIC (Thermo Scientific) 2.6  $\mu\text{m}$ , 100 x 2.1 mm i.d.; Hypersil Gold aQ (Thermo Scientific) 1.9  
229  $\mu\text{m}$ , 100 x 2.1 mm i.d.; Zic-pHILIC (Merck Millipore) 5.0  $\mu\text{m}$ , 150 x 4.6 mm i.d. and Acquity BEH  
230 HILIC (Waters) 1.7  $\mu\text{m}$ , 100 x 2.1 mm. Peak shape was not optimal with the Hypercarb, Accucore  
231 Urea HILIC and Hypersil Gold aQ columns. Moreover, the Zic-pHILIC column did not allow to  
232 separate ACh and  $\gamma$ -butyrobetain in the tested conditions. The Acquity BEH HILIC column was  
233 therefore retained for the analytical method development and validation. With this latest column,  
234 retention time for ACh, Ch and  $\gamma$ -butyrobetain were 5.9, 6.9 and 10.6 min, respectively.

235

#### 236 *Mobile phase optimization*

237 The pH of the mobile phase was optimised in order to separate ACh and  $\gamma$ -butyrobetain. The  
238 following pH were tested: 3.0 and 5.5. Only pH 5.5 allowed to separate ACh and  $\gamma$ -butyrobetain,  
239 retention time of ACh and  $\gamma$ -butyrobetain at pH 3.0 were both 5.1 min.

240

#### 241 *MS/MS optimization*

242 ACh, Ch,  $\gamma$ -butyrobetain and ACh-d<sub>16</sub> are quaternary ammoniums, therefore ionized in  
243 physiological conditions. In line with this, the precursor ions for the detection of ACh, Ch,  $\gamma$ -  
244 butyrobetain and ACh-d<sub>16</sub> in the positive ionization mode were  $m/z = 146.1$  for both ACh and  $\gamma$ -  
245 butyrobetain, 104.4 for Ch and 162.2 for ACh-d<sub>16</sub>. Two products ions were observed during the

246 fragmentation of each precursor ion. The most intense MRM transitions were used for the  
247 quantification of each compound and IS, whereas the other transitions for each compound were  
248 used for confirmation. Representative chromatograms of the analysis of calibration standard and  
249 patient samples are shown in Figures 1 and 2.

250

## 251 **Method validation**

### 252 *Selectivity and carry over*

253 For ACh and Ch, the mean of the ratio between peak area in samples spiked at the LLOQ and blank  
254 samples was 13 and 67, respectively, while the mean was greater than 10000 for the internal  
255 standard, demonstrating the selectivity for each molecule. The peak area of ACh, Ch and internal  
256 standards in blank samples injected after the highest CS were lower than 5% of the peak area at the  
257 LLOQ ( $n = 5$ ), demonstrating the absence of carry-over for all analytes.

258

### 259 *Lower limit of quantification*

260 The LLOQ was set at 5 ng/L (= 34.2 pmol/L) and 5 µg/L for ACh and Ch, respectively, providing a  
261 signal superior to 5 times the signal of a blank sample. Intra- and interday precisions were 5.3% and  
262 10.2% respectively, and intra- and interday accuracies 96.2-117.2% and 113.6% respectively.

263 Results are summarized in Tables 1 and 2 for ACh and Ch, respectively. This method has excellent  
264 sensitivity since the LLOQ for ACh at 5 ng/L is equivalent to 0.16 fmol on column, increasing by  
265 1.5-fold the lowest sensitivity described to date [15].

266

### 267 *Calibration curve*

268 The calibration range was defined by eight calibration standards relevant with ACh and Ch  
269 concentrations described in human CSF samples. The visual inspection of the graphs plotting the  
270 peak area ratios of each molecule to the internal standard versus concentration suggested the

271 linearity of the response, justifying the choice of an ordinary least square model to fit the data.  
272 Since the pattern of residuals plotted as a function of the concentration was heterogeneous,  
273 demonstrating heteroscedasticity, a weighted least square regression model was applied with a  $1/x$   
274 weighing factor. Mean fitted equation obtained from six curves were  $y = 0.00020 (\pm 0.00001) x +$   
275  $0.00049 (\pm 0.00045)$  (mean  $\pm$  SD) for ACh and  $y = 0.07356 (\pm 0.00394) x - 0.20125 (\pm 0.03619)$  for  
276 Ch; mean least squares linear regression correlation coefficient ( $r^2$ ) were  $0.9995 (\pm 0.0004)$  and  
277  $0.9994 (\pm 0.0004)$ , respectively. The interday CVs of the back-calculated concentrations of the eight  
278 calibration standards ranged from 0.6 to 6.6% and the mean bias ranged from -7.4 to 12.9% for both  
279 ACh and Ch.

280

#### 281 *Accuracy and precision*

282 Intraday and interday precision and accuracy of the QC samples at the 3 concentration levels are  
283 summarized in Tables 1 and 2 for ACh and Ch, respectively. Intraday precision ranged from 1.8 to  
284 3.8% with an accuracy ranging from 85.2 to 105.4%. Interday precision ranged from 2.1 to 7.9%  
285 with an accuracy from 86.3 to 104.6%. As a further assessment of selectivity,  $\gamma$ -butyrobetain was  
286 also spiked at 3 levels in QC samples and accuracy and precision data for this compound are shown  
287 in supplemental Table 1. Altogether, the results demonstrate the absence of interferences from  $\gamma$ -  
288 butyrobetain for the quantitative analysis of ACh and Ch. The developed method is thus highly  
289 selective, precise and accurate in a wide range of concentrations for both ACh and Ch.

290

#### 291 *Precipitation recovery, matrix effect and overall recovery*

292 Precipitation recovery, matrix effect and overall recovery results are summarized in Table 3. The  
293 precipitation recovery was between 107.4 and 140.0% for ACh, Ch and ACh- $d_{16}$ . The matrix effect  
294 was concentration-independent since the matrix factor was between 94.5 and 106.8% at the low QC  
295 level and between 73.5 and 79.7% for the high QC level. The IS-normalized matrix effect was in

296 the range 92.8 – 108.6%, demonstrating the usefulness of the deuterated internal standard. The  
297 coefficients of variation of the IS-normalized matrix effect for ACh and Ch were lower than 15%  
298 for the two QC levels. The overall recovery, which combines the evaluation of precipitation  
299 recovery and matrix effect is in perfect agreement with the above-mentioned findings.  
300 Precipitation recovery and matrix effect were respectively 91.7 and 83.4% for ACh-d<sub>16</sub> in CSF  
301 samples (n = 6) demonstrating PBS is an appropriate surrogate matrix. The surrogate matrix of the  
302 present study was chosen and validated as recommended by a discussion group on selection strategy  
303 of surrogate matrix to quantify endogenous substances, which recommended the use of water, saline  
304 or buffer solutions such as PBS as surrogate matrix and detailed the validation steps required [30].  
305 The validity of the selected matrix was therefore assessed by evaluating selectivity, accuracy and  
306 precision for all analytes in the surrogate matrix, but also by comparing matrix effect and extraction  
307 recovery for acetylcholine in the surrogate matrix to those observed for the deuterated analyte in the  
308 original matrix. The results complied with requirements, in particular with regard to accuracy and  
309 precision, and did not reveal concerns on solubility, adsorption or stability in the surrogate matrix.

310

### 311 *Stability*

312 The stability in QC samples after storage for 3 months at -80°C was evaluated and the differences  
313 between the measured and nominal concentrations were in the range -5.2 – 13.9% (mean = 6.6%)  
314 for Ach and Ch. One of the limitations of this stability study consists in the analysis of QC samples.  
315 Although these storage conditions are similar to those in previous reports, [6, 7, 11, 31-36] these  
316 results should be confirmed with reanalysis of patient samples after long-term storage.  
317 The stability of processed QC samples was evaluated by reinjection (with a new calibration curve)  
318 of the samples kept at +4°C in the autosampler after 24hrs for the 3 QC levels. All the differences  
319 between the measured and nominal concentrations were in the range -2.4 – 6.1% (mean = 1.3%) for  
320 Ach and Ch, showing the stability of processed samples for 24hrs in the autosampler.

321

322 **Analysis of study samples**

323 ACh, Ch and  $\gamma$ -butyrobetain concentrations measured in 10 CSF patient samples are shown in  
324 Figure 3. Concentrations were in the range 10.0 - 27.7 ng/L for ACh ( $n = 9$  patients, not detectable  
325 in the last patient), 208.5 - 382.5  $\mu\text{g/L}$  for Ch and 1.9 - 5.1  $\mu\text{g/L}$  for  $\gamma$ -butyrobetain, highlighting the  
326 usefulness of a separation between ACh and  $\gamma$ -butyrobetain and a very sensitive analytical method  
327 for ACh.

328



329 **CONCLUSION**

330 A method for the simultaneous quantitation of acetylcholine and choline in human CSF samples is  
331 described, adding the following advantages when compared to previously published methods: (1)  
332 very high sensitivity (1.6 times greater than previous methods), (2) high selectivity towards other  
333 endogenous compounds (3) full method validation according to the EMA and FDA guideline and  
334 (4) validation of the suitability of the method with clinical samples. This method will allow to  
335 determine ACh concentrations in CSF samples in order to better characterize the cholinergic  
336 neurotransmission in patients and the effects of drugs acting on this system and to improve  
337 medicine personalization.

338

339 **ACKNOWLEDGEMENTS**

340 None

341

342 **AUTHOR CONTRIBUTIONS**

343 All the authors have accepted responsibility for the entire content of this submitted manuscript and  
344 approved submission.

345

346 **RESEARCH FUNDING**

347 The clinical study was funded by CRC APHP 2016 “CHOLINE”.

348

349 **DISCLOSURE OF CONFLICTS OF INTEREST**

350 The authors declare no conflict of interest.

351

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## TABLES

**Table 1:** Accuracy and precision data for acetylcholine.

QC concentration (ng/L)	Concentration found each day (mean $\pm$ SEM)	Intraday ( <i>n</i> = 6)		Interday ( <i>n</i> = 6)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
5.0 (LLOQ)	D <sub>1</sub> : 4.8 $\pm$ 0.09	5.3	96.2-104.3	10.2	100.8
	D <sub>2</sub> : 5.2 $\pm$ 0.09				
	D <sub>3</sub> : 5.1 $\pm$ 0.1				
12.0	D <sub>1</sub> : 11.6 $\pm$ 0.2	3.3	92.5-97.0	6.3	94.2
	D <sub>2</sub> : 11.1 $\pm$ 0.01				
	D <sub>3</sub> : 11.2 $\pm$ 0.2				
40.0	D <sub>1</sub> : 42.1 $\pm$ 0.4	3.8	102.9-105.4	3.3	103.8
	D <sub>2</sub> : 41.2 $\pm$ 0.3				
	D <sub>3</sub> : 41.2 $\pm$ 1.0				
175.0	D <sub>1</sub> : 150.4 $\pm$ 1.6	1.8	85.2-87.7	3.6	86.3
	D <sub>2</sub> : 149.2 $\pm$ 0.7				
	D <sub>3</sub> : 153.4 $\pm$ 0.8				



**Table 2:** Accuracy and precision data for choline.

QC concentration (µg/L)	Concentration found each day (mean ± SEM)	Intraday ( <i>n</i> = 6)		Interday ( <i>n</i> = 6)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
5.0 (LLOQ)	D <sub>1</sub> : 5.6 ± 0.07	2.0	111.4-117.2	6.7	113.6
	D <sub>2</sub> : 5.9 ± 0.02				
	D <sub>3</sub> : 5.6 ± 0.04				
15.0	D <sub>1</sub> : 14.1 ± 0.1	1.9	89.5-94.1	6.2	91.7
	D <sub>2</sub> : 13.7 ± 0.07				
	D <sub>3</sub> : 13.4 ± 0.1				
300.0	D <sub>1</sub> : 314.8 ± 2.3	2.1	103.6-105.3	2.1	104.6
	D <sub>2</sub> : 316.0 ± 3.6				
	D <sub>3</sub> : 310.8 ± 1.9				
800.0	D <sub>1</sub> : 720.6 ± 5.2	1.8	90.1-96.0	7.9	92.7
	D <sub>2</sub> : 737.2 ± 5.1				
	D <sub>3</sub> : 767.7 ± 6.3				

**Table 3:** Precipitation recovery, matrix effect and overall method recovery for acetylcholine, choline and internal standard (mean  $\pm$  SEM (CV) (%)).

	Precipitation recovery	Matrix factor	IS-normalized matrix factor	Overall recovery
<b>Acetylcholine</b>				
12.0 ng/L ( <i>n</i> = 6)	109.9 $\pm$ 4.2 (9.3)	106.8 $\pm$ 7.4 (17.1)	108.6 $\pm$ 3.4 (7.6)	116.0 $\pm$ 4.5 (9.6)
175.0 ng/L ( <i>n</i> = 6)	114.2 $\pm$ 2.5 (5.3)	78.1 $\pm$ 2.2 (6.8)	98.7 $\pm$ 4.3 (10.7)	89.0 $\pm$ 0.9 (2.4)
<b>Choline</b>				
15.0 $\mu$ g/L ( <i>n</i> = 6)	107.4 $\pm$ 5.2 (11.8)	94.5 $\pm$ 5.8 (15.1)	96.3 $\pm$ 3.1 (8.0)	100.2 $\pm$ 2.7 (6.5)
800.0 $\mu$ g/L ( <i>n</i> = 6)	110.6 $\pm$ 4.0 (9.0)	73.5 $\pm$ 2.0 (6.5)	92.8 $\pm$ 3.9 (10.4)	80.9 $\pm$ 1.6 (4.9)
<b>Internal standard</b>				
100 ng/L ( <i>n</i> = 12)	137.3 $\pm$ 7.1 (17.8)	89.1 $\pm$ 4.3 (16.8)	NA	120.2 $\pm$ 5.0 (14.5)

## FIGURE CAPTIONS

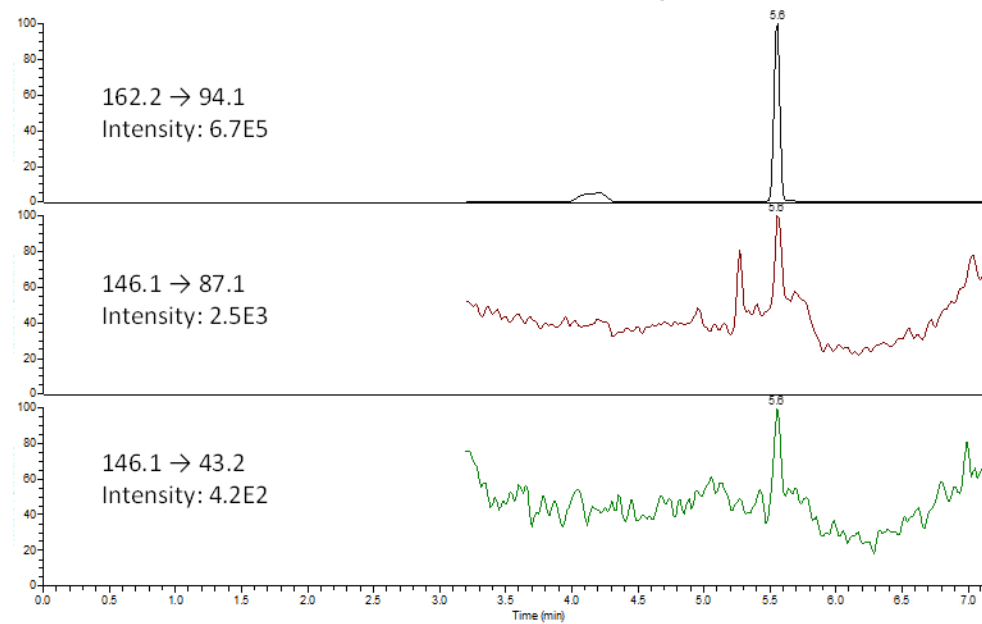
**Figure 1: Chromatograms of acetylcholine and internal standard.** Representative LC-MS/MS chromatograms of acetylcholine (2 lower graphs in each panel) and internal standard (upper graph panel) obtained after the analysis of a calibration standards at the LLOQ (A), at the ULOQ (B), of a blank PBS sample (C) and of a patient CSF sample (D).

**Figure 2: Chromatograms of  $\gamma$ -butyrobetain and choline.** Representative LC-MS/MS chromatograms of  $\gamma$ -butyrobetain (2 upper graphs in each panel) and choline (2 lower graphs in each panel) obtained after the analysis of a calibration standards at the LLOQ (A), at the ULOQ (B), of a blank PBS sample (C) and of a patient CSF sample (D).

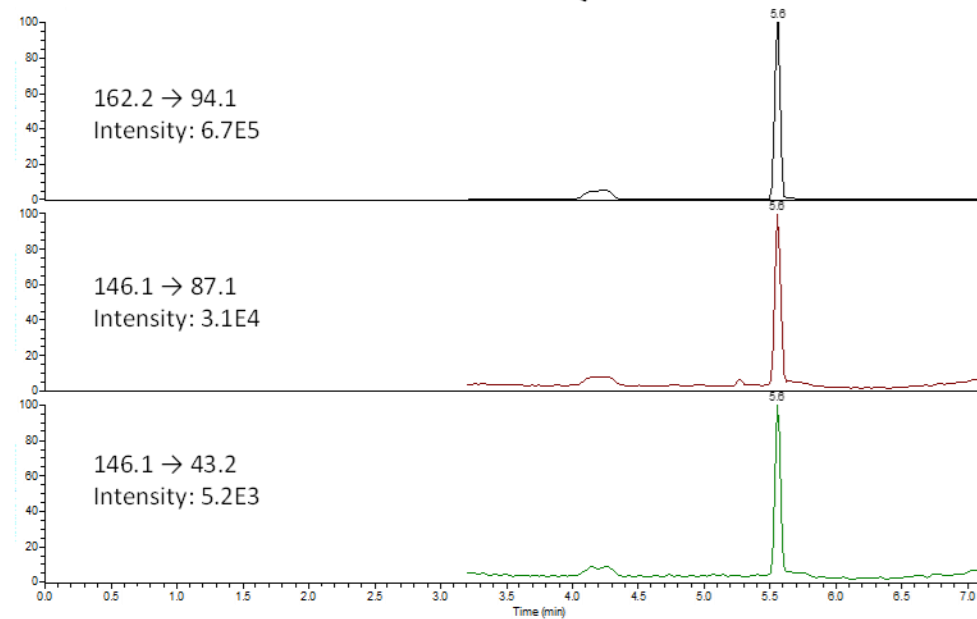
**Figure 3: Human CSF samples analysis.** Acetylcholine,  $\gamma$ -butyrobetain and choline in CSF samples from 10 patients.

Figure 1

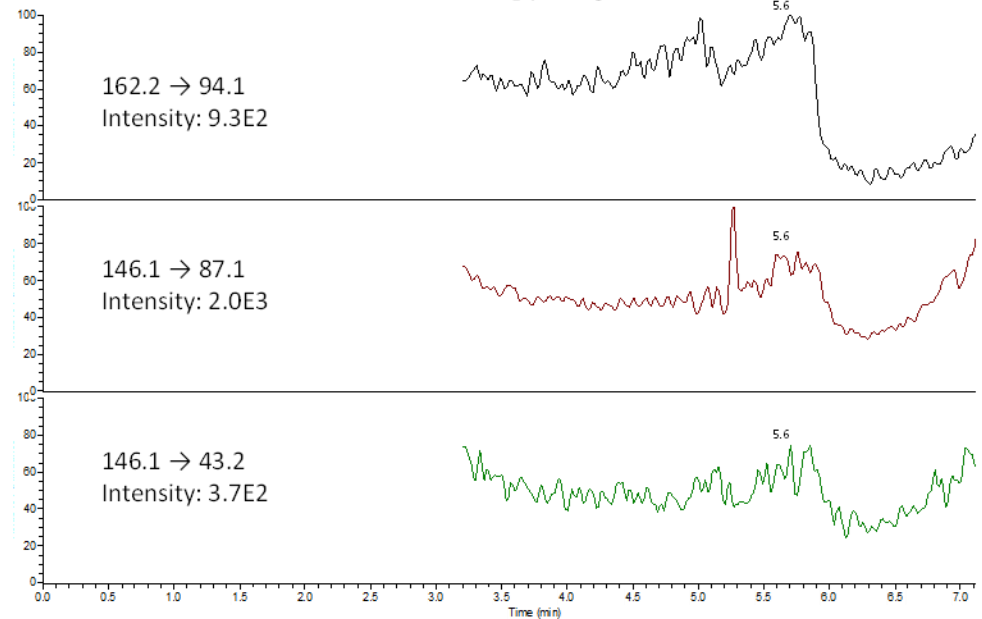
A. LLOQ



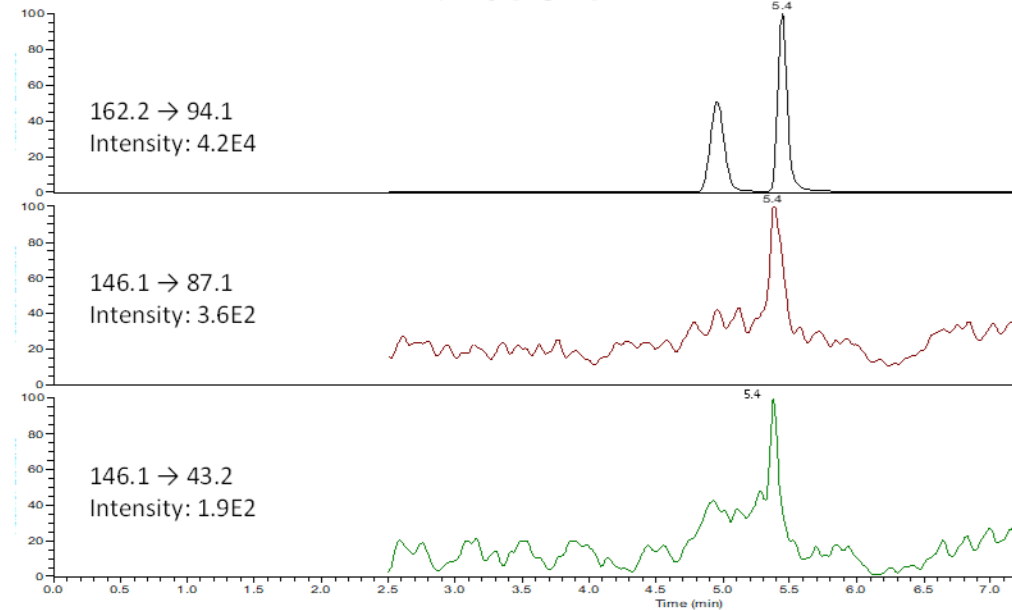
B. ULOQ



C. Blank



D. Patient



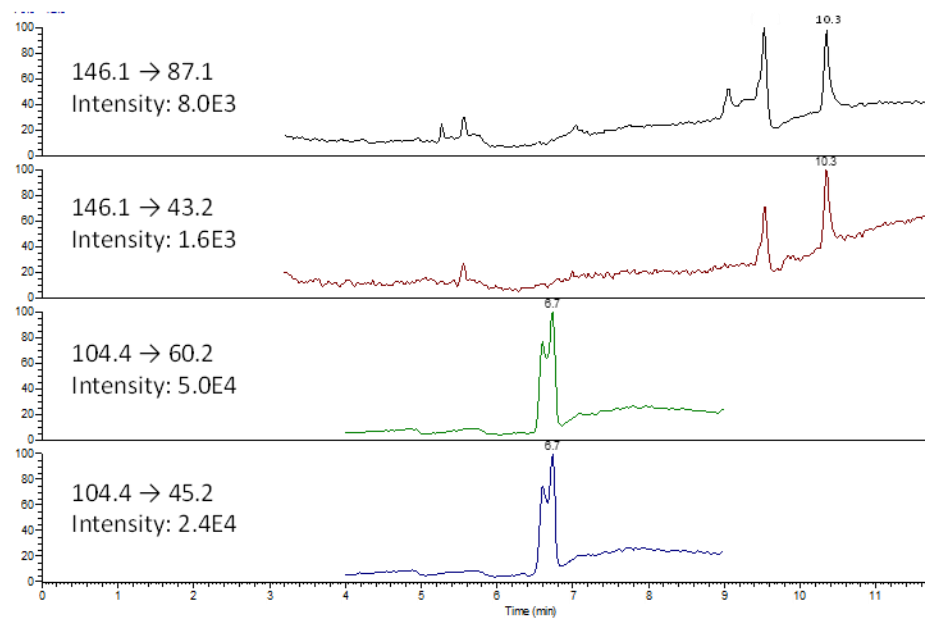
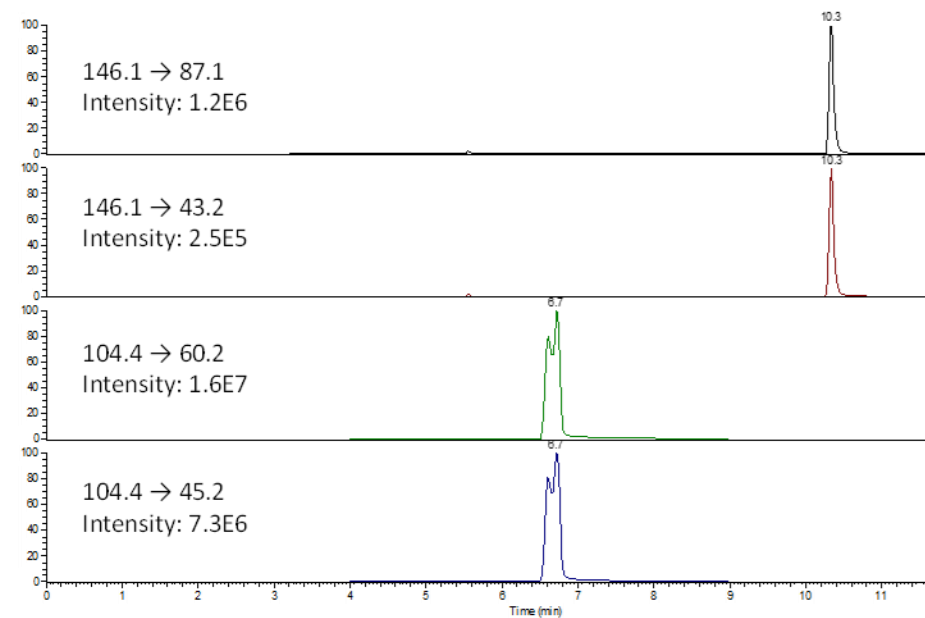
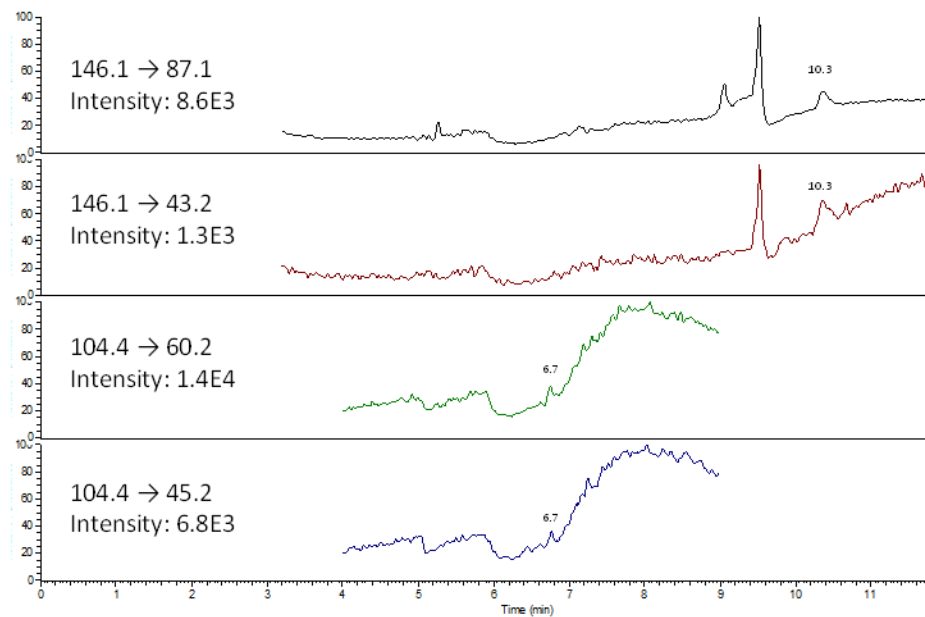
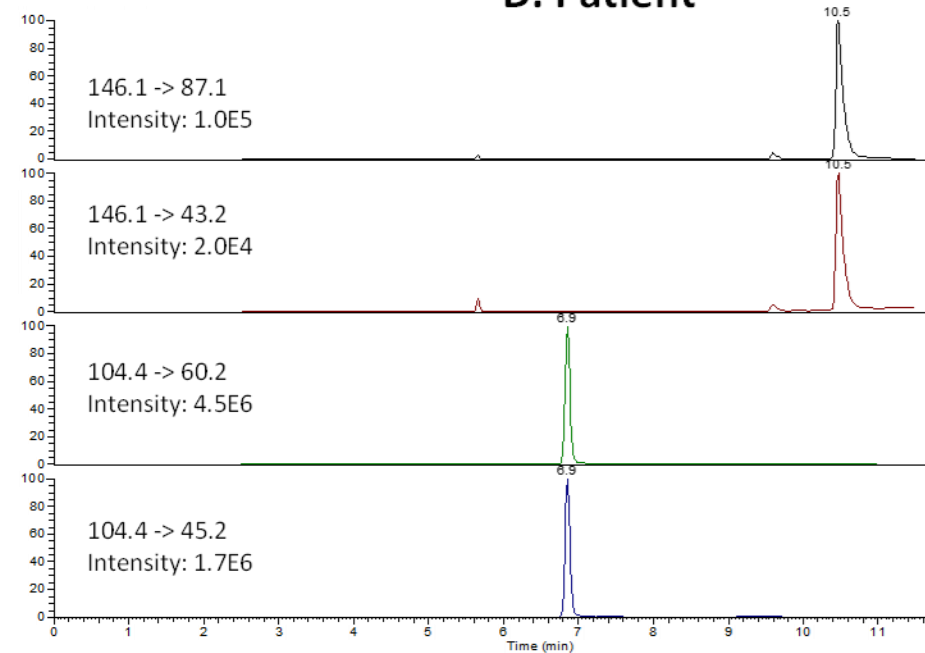
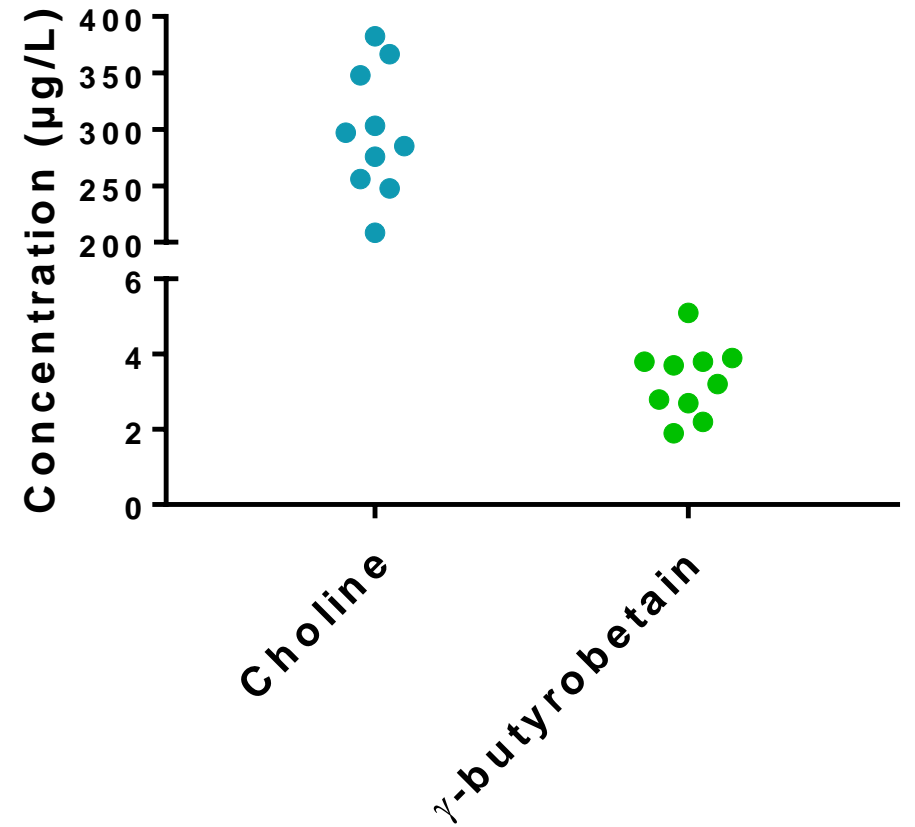
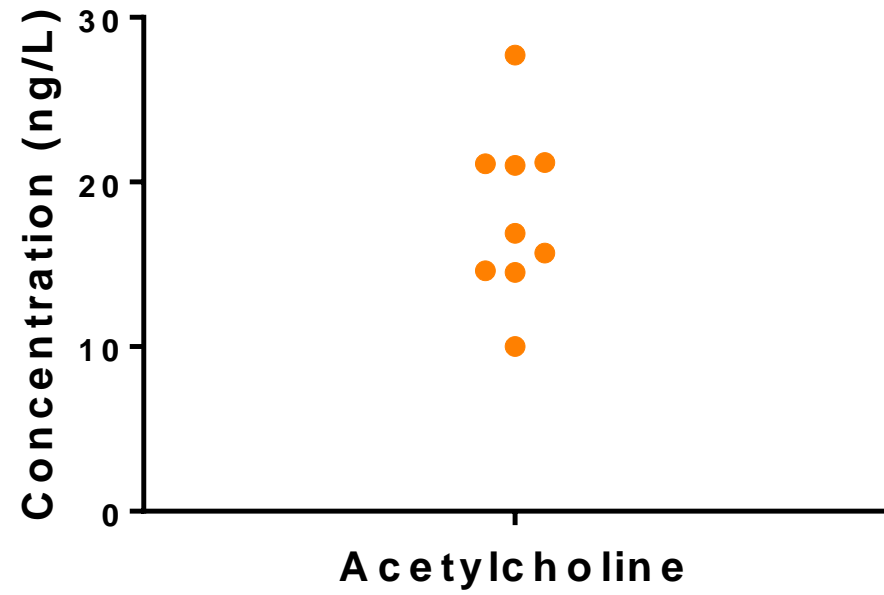
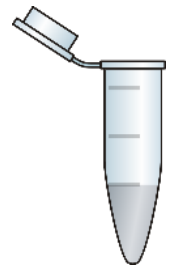
**Figure 2****A. LLOQ****B. ULOQ****C. Blank****D. Patient**

Figure 3





**Cerebrospinal fluid**



**Precipitation**



**LC-MS/MS analysis**

