Westfälische Wilhelms-Universität Münster

Nils Helge Schebb[1], Daniel Fischer[2], Josef Krieglstein[2], Susanne Klumpp[2] and Uwe Karst[1]

[1] University of Münster, Institute of Inorganic and Analytical Chemistry
Corrensstr. 30, 48149 Münster, Germany

[2] University of Münster, Institute of Pharmaceutical and Medicinal Chemistry
Hittorfstr. 58-62, 48149 Münster, Germany

Institut für Anorganische und Analytische Chemie

Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry for the Determination of Acetylcholine in Cultured Cells

Background:

Acetylcholine (ACh, Fig.1) is an essential messenger in neurotransmission in both the peripheral and central nervous system (CNS). It has an effect on alertness, memory and learning. Decreased levels of ACh are found in patients with Alzheimer's disease. In order to develop drugs against this disease, the ongoing processes in the neuronal cell, leading to a depletion of ACh have to be understood. Cell cultures are a common and useful model to investigate the molecular biology of tissues. To investigate the role of the signal cascades or the effect of potential drugs on the ACh synthesis in cells, the ACh concentration in the cells has to be determined. Therefore, we developed a new LC-MS/MS method for the fast measurement of ACh concentration in cell homogenates .

Approach:

- Cells were lysed directly in the HPLC solvent. By addition of neostigmin, the cleavage of ACh by cellular esterase is inhibited. After centrifugation no further sample preparation is needed.
- To compensate for ion-suppression effects of the resulting complex matrix d_4-acetylcholine (Fig.1) is used as internal standard (ISTD).
- To separate ACh from the most components of the cell extract, hydrophobic interaction liquid chromatography (HILIC) is used. Separations are carried out isocratically with 80% ACN in 0.1% formic acid to avoid long equilibration times.
- Postive electrospray ionisation tandem mass spectrometry (ESI(+)-MS/MS) is applied for detection using the transition of 146→87 amu for ACh and 150→90 amu for the ISTD.

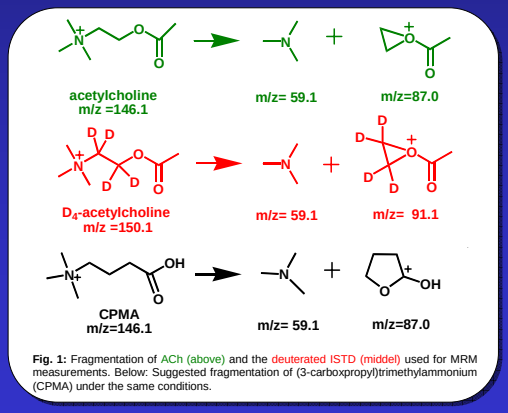
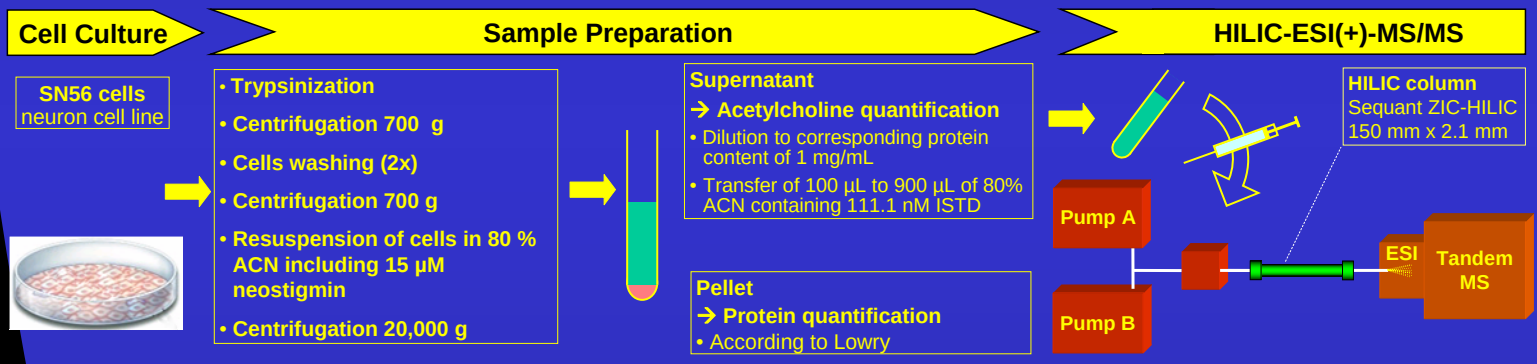


Fig. 1: Fragmentation of ACh (above) and the deuterated ISTD (middel) used for MRM measurements. Below: Suggested fragmentation of (3-carboxypropyl)trimethylammonium (CPMA) under the same conditions.



Results:

- Fast separation of ACh from neostigmin (peak 2), choline (peak 3) and the constituents not being retained (Peak 1) of the cell lysate (Fig. 2).
- SN56 cell lysate contains small amounts of (3-carboxypropyl)trimethylammonium (CPMA, Fig.1), a constituion isomer of ACh, showing the same MRM transition as ACh. CPMA (Peak 6) is baseline separated from ACh and does not infere with the quantification (Fig.2).

The developed method provides:

- Analysis time of less than 10 min per sample.
- Wide linear range of three decades (Table 1).
- Low LOD (0.3 nM) and a low LOQ (1.5 nM).
- High inter- and intraday reproducibily of the measured ACh concentration in cell samples (Table 2).

Table 1: Precision and accuracy of calibration standards. The measured ratios of the peak area of ACh to the peak area of ISTD (100 nM) of a typical calibration curve is shown. The relative standard deviation (RSD) is based on triple injections. The calculated content by the regression function (R^2 = 0.997) is divided by the concentration to determine the accuracy for each standard.

Concentration nM	Ratio ACh/ ISTD	RSD [%]	Calculated concentration nM	Accuracy [%]
2	0.022	7.52	1.87	93.7
10	0.102	2.03	10.01	100.1
20	0.195	2.33	19.48	97.4
100	0.969	1.28	98.02	98.0
200	1.945	1.24	197.16	98.6
1000	10.118	2.78	1026.86	102.7
2000	20.453	1.65	2076.12	103.8
10000	98.937	2.82	10044.07	100.4

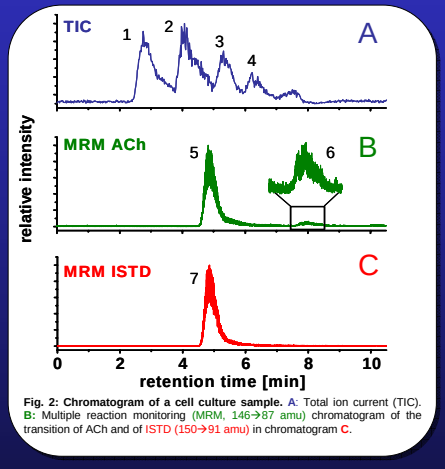


Fig. 2: Chromatogram of a cell culture sample. A: Total ion current (TIC). B: Multiple reaction monitoring (MRM, 146→87 amu) chromatogram of the transition of ACh and of ISTD (150→91 amu) in chromatogram C.

Table 2: Determined ACh concentrations in SN56 cells. ACh has been determined for two passages in 15 independent cell lysates. The RSD is based on triple injections. The interday deviation was calculated by the measurement of the samples on three different days.

Cell lysate	day 1 ACh [pmol/mg prot.]	RSD [%]	day 2 ACh [pmol/mg prot.]	RSD [%]	day 3 ACh [pmol/mg prot.]	RSD [%]	mean ACh [pmol/mg prot]	interday RSD [%]
Passage # 32								
1	1220	0.53	1222	0.01	1229	0.93	1223	0.38
2	1110	1.68	1143	1.06	1102	1.36	1118	1.98
3	1183	0.44	1180	0.24	1182	2.29	1182	0.10
4	1081	0.68	1099	0.35	1106	0.42	1095	1.21
5	1271	0.79	1251	1.18	1299	1.27	1274	1.90
6	1113	1.02	1122	2.41	1101	0.42	1112	0.92
7	1226	1.21	1170	0.43	1197	1.96	1198	2.33
8	1231	0.30	1223	0.93	1189	0.54	1214	1.85
9	1289	0.88	1283	1.69	1282	0.97	1285	0.27
10	1175	0.83	1232	1.24	1183	0.69	1197	2.57
Average	**1190**	**0.84**	**1193**	**0.95**	**1187**	**1.08**	**1190**	**1.35**
Passage # 33								
1	1281	0.84	1198	1.19	1198	0.87	1225	3.93
2	1290	1.41	1252	2.09	1271	0.84	1271	1.52
3	1439	0.58	1419	0.78	1404	1.16	1421	1.21
4	1525	0.24	1471	0.91	1470	0.49	1488	2.12
5	1362	1.90	1398	0.82	1347	0.52	1369	1.91
Average	**1379**	**0.99**	**1347**	**1.16**	**1338**	**0.78**	**1355**	**2.14**
					overall average		**1272**	**1.75**

Outlook:

- Investigation regarding ACh synthesis in tranfected SN56 cells:
  - Overexpression and downregulation of protein-histidine phosphatase (PHP)
  - Clarify the influence of PHP on regulation of cellular ACh synthesis

Literatur:

N.H. Schebb, D. Fischer, E-M. Hain, H. Hayen, J. Krieglstein, S. Klumpp, U.Karst: Fast sample preparation and liquid chromatography–tandem mass spectrometry method for assaying cell lysate acetylcholine. J. Chrom. A. (**2008**) 1183, 100-107.

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