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Exorphin Peptides in Urine with HPLC-MS/MS Detection

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Abstract

Exorphins have been found in urine from individuals diagnosed with autism spectrum disorders by HPLC techniques. However, several studies, using sophisticated analytical techniques, have reported negative findings. This made it necessary to improve our methods. The sample stability during transport and storage and the pre-analytical treatment of urines was improved by peptidase inhibition and solid phase extraction respectively. HPLC –mass spectrometry with collision induced fragmentation (MS/MS) was developed to a routine method. Adding citric acid or acetic acid to urines prevented room temperature dependent break-down during transport and storage. Solid phase extraction of the exorphins cleaned up the mass spectrometry results.
The presence of exorphins could be demonstrated in urines from autistic children.

Keywords: *Autism, exorphins, mass spectrometry, MS/MS peptidases*

1. Introduction

Exorphins have been found in urine from individuals diagnosed with different psychiatric disorders by means of HPLC (high performance liquid chromatography), immune techniques and receptor displacement assay [1-7]. However, several studies based on the use of sophisticated mass spectrometric techniques [8, 9] have induced skepticism to earlier findings.
We wish to report the fast peptide degradation when urine is kept at room temperature. The addition of boric acid has been used by some to prevent peptide degradation, while we routinely used fast freezing of the samples. Inhibitors that prevent the peptide breakdown have been found. As an example MS/MS mass fragmentation, identification and quantification of β-casomorphin 1-4(bovine) is reported in this work. A routine method for peptide analysis has been developed and implemented in our laboratory. This procedure has been applied to all the exorphins listed.

2. Methods

2.1 Peptides, chemicals and materials

Peptide standards of bovine β-casomorphin 1-3(Y-P-F), β-casomorphin 1-4(Y-P-F-P), β-casomorphin 1-4 amide, β-casomorphin 1-5(Y-P-F-P-G), β-casomorphin 1-5 amide, β -casomorphin 1-7(Y-P-F-P-G-P), β-casomorphin 1-8(Y-P-F-P-G-P-I), gluten exorphin A4(G-Y-Y-P), gluten exorphin A5(G-Y-Y-P-T), gluten exorphin B4(Y-G-G-W), gluten exorphin B5(Y-G-G-W-L) and gluten exorphin C(Y-P-I-S-L) were purchased from Bachem, Switzerland with > 97% purity. Internal standards were isotope labelled β-casomorphin 1-4 (13C9, 15N) and gluten exorphin B4 (13C2, 15N) and were purchased from Bachem, Switzerland with > 99% purity. HPLC-grade deionized water, acetonitrile, isopropanol, formic acid, acetic acid, citric acid, boric acid, hydrochloric acid, trifluoroacetic acid were obtained from Sigma-Aldrich. Overmolded Millex 13 mm, 0.22 µm PVDF syringe filters were purchased from Merck Millipore (Oslo, Norway). Supelco LC-CN SPE and Sepak C-18 cartridges were bought from Sigma-Aldrich (Norway).

2.2 Instrumentation and data acquisition

Peptide separation was performed on a HPLC-instrument from Agilent Technologies (Hewlett Packard 1100). Detection of peptides was performed on an ESI-MS-MS 6410 Triple Quad mass spectrometer from Agilent Technologies – software used for data acquisition and processing was Masshunter Workstation (version B.03.01 for data acquisition, B.04.00 for qualitative analysis, and B.05.00 for quantitative analysis), delivered by Agilent Technologies. All peptides were detected using positive ionization mode with a collision gas temperature at 300 °C, MRM transitions were optimized using peptide standards.

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2.3 Peptide standards

Each peptide was individually dissolved in HPLC-grade water with 0.1% v/v formic acid. The concentration of the individual peptide stock solution was 10 µM. A stock solution containing all peptides was also prepared to a concentration of 10 000 ng/ml. Sample urines were spiked with peptide stock solutions to a final concentration of 500 nMolar, and filtered through 0.22 µm PVDF syringe filter before HPLC-MS/MS analysis.
Spiked urine from donors was preserved in different acids for comparison. Normal urine was analyzed before spiking to exclude any endogenous analytes that could be present. Each urine sample was then divided equally into 6 vials and the acids to be tested were added individually to each of the vials. The acids added were boric -, hydrochloric -, acetic -, formic -, trifluoracetic- and citric acid at a concentration of 0.1% by volume. Each vial was finally spiked to 500nmolar with the same concentration of β-casomorphin 1-4(bovine), here abbreviated to CM1-4.
The inhibiting effect of the individual acids on peptide breakdown was also tested on a spiked urine sample containing all the peptides. This sample was compared to a spiked urine sample containing no inhibitors.
Observation of peptide stability in urine was performed by storing spiked urine at room temperature overnight. The urine was then analyzed and the peptide levels were compared to those of a freshly spiked urine sample. The urine samples studied for peptide breakdown were not concentrated by solid phase extraction.

2.4 Solid phase extraction (SPE)

We used 1 ml C18 reverse phase cartridges with 100 mg sorbent. The column sorbent was activated by 1.0 ml 100 % acetonitril and readied for use with 1% by volume formic acid. A vacuum manifold connected to a water aspirator was used to treat each 1 ml sample. Elution of peptides was performed with 1.0 mL of 100% acetonitrile containing 0.1% (v/v) formic acid. Eluted sample were evaporated in a nitrogen stream for approximately 15 minutes, and re dissolved in 200 µl of HPLC-grade water with 0.1 % (v/v) formic acid.

2.5 Peptide separation and detection

Peptide separation was performed with an ACE C-18 reverse phase column of dimension 150×4.6 mm, high purity silica and high carbon load with a particle diameter of 5 or 3µm. A guard column with the same material was routinely used.

The mobile phase consisted of a buffered solution containing A - HPLC-grade water with 0,1% w/v ammonium formate and 0,1% v/v formic acid, and B - 30% isopropanol and 70% acetonitrile containing 0,1% v/v formic acid. The mobile phase flow rate was set at 1.0 mL/min. For separating the peptides the gradient program presented in Table 1 was used.

Table 1: HPLC-gradient program used to separate peptides in spiked urine samples.

Time (min)	Eluent A (%)	Eluent B (%)
0	86	14
7	80	20
8	35	65
12	35	65
13	86	14
17	86	14

Total time of analysis was optimally 17 min. ESI-MS variables such as collision energy and fragmentor voltage and MRM transitions (multiple reaction monitoring) for the peptides were optimized for each peptide. Peptides were detected using positive ionization mode. MRM transitions used for the casein and gluten derived peptides are presented in Table 2 and 3 respectively.

Table 2: MRM-transitions for opioids from casein

Peptide	Structure	Mother ion	Quantitation ion* Qualification ion m/z	Collision energy (eV)	Fragmentation energy (V)
Cm1-3	YPF	426.2	70.1* 263.1	36 20	140
Cm1-4	YPFP	523.26	70.1* 136.1	60 44	170
Cm1-4 A	YPFPnh$_2$	522.26	70.1* 136.1	64 48	140
Cm1-5	YPFPG	580.31	70.1* 173.1	72 28	175
Cm1-5A	YPFPGnh$_2$	579.31	70.1* 172.1	68 28	170
Cm1-7	YPFPGP	790.31	70.1* 229.1	96 44	225
Cm1-8	YPFPGPI	887.5	70.1* 155.3	108 72	250
Cm14IS	#	533.26	70.1* 136.1	56 44	145

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#IS=Internal standard, $C_{19}{}^{13}C_9H_{34}N_3{}^{15}NO_6$. *marks the quantification ion. The other fragment is used as qualification ion. A is C terminal amide or nh_2.

Table 3: MRM transitions for opioids from gluten.

Peptide	Structure	Mother ion	Quantitation ion* Qualification ion m/z	Collision energy (eV)	Fragmentation energy (V)
Gluten exorphin A4	GYYP	499,5	91,1* 136,1	96 36	125
Gluten exorphin A5	GYYPT	600,27	217,1* 136.1	20 52	140
Gluten exorphin B4	YGGW	482,2	188,1* 136.1	34 38	157
Gluten exorphin B5	YGGWL	595,29	159,1* 132,1	48 80	165
Gluten exorphin C	YPISL	592,34	70,1* 136,1	60 52	175
Gluten exorphin B4*, isotope labelled		485,21	188,0* 136,1	36 44	140

*Internal standard, $C_{22}{}^{13}C_2H_{27}N_4{}^{15}NO_6$

3. Results

Sample urine spiked with a mix containing all peptides was found to be completely degraded within 1 day at room temperature when incubated without addition of acid. When different types of acids were added to the spiked urine samples, variable degrees of peptide stability were observed.

3.1 Example of observed peptide degradation

The urine sample spiked with bovine β- Casomorphin 1-4 (CM1-4) was analyzed on the same day it was prepared, and incubated in room temperature and analyzed the next day, and finally 3 days after spiking. Figure 1 shows effect of incubating urine spiked with CM1-4 over a period of 3 days in room temperature. Fragment detected around 4 minutes was predicted to be proline-phenylalanine according to precursor mass and fragmented ammonium ions. The Phe-Pro fragment increased and the level of CM1-4 decreased over time. The mass of the degraded peptide CM1-4 was found to be m/z = 263.1. This ion fragment was predicted to consist of proline and phenylalanine. MRM-transitions for Pro-Phe were 263.1 > 70.1 and 263.1 > 120.0, both transitions were performed with collision energy at 25 eV and fragmentation voltage at 135 V. Similar results were found for the other opioids in different spiked urines.

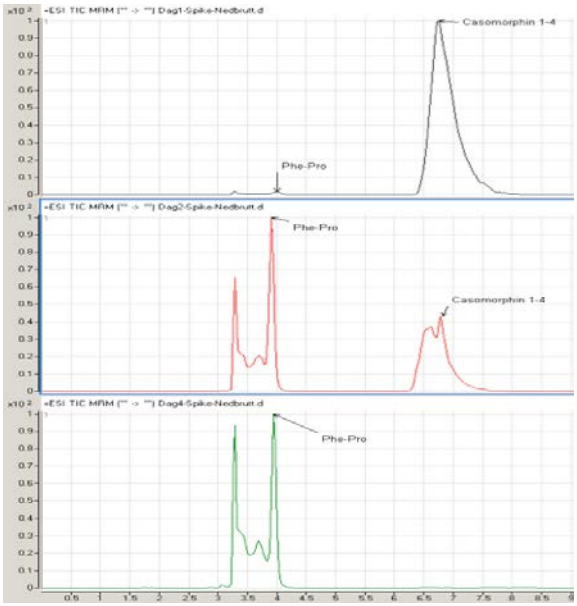

Figure 1. Degradation of bovine β-casomorphin1-4(Y-P-F-P) at room temperature for 3 days. Top trace is the initial -peptide level, middle trace is from the next day and bottom trace in after three days at room temperature.

The acids used as inhibitors of peptide degradation were shown to have different effects. Boric -, hydrochloric -, trifluoracetic - and formic acid had no effect on the degradation. However, acetic and citric acid had a significant effect on the breakdown, probably inhibiting peptidase(s). The decrease of CM1-4 or peptide mix with added acetic acid or citric acid was inhibited when comparing peptide abundance for the incubated urine spiked with standards. If the urine was collected over citric acid to a final concentration of 0.2M, it was stable at room temperature for 14 days.

3.2 Peptide detection in urine

Several urine samples were spiked with a mix containing all peptides to determine the effect of solid phase extraction using Sepak C18 cartridges and the conditioning/washing procedure as outlined for the cartridges. Urine from co-workers was spiked with a peptide mix, samples were extracted and the recovery of peptides measured. We found the minimum amount of

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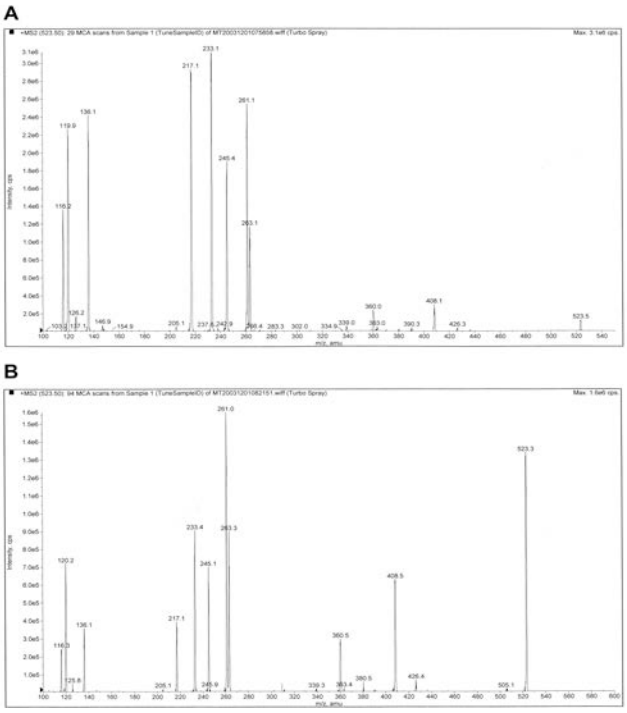
peptides detected after application to Sepak C-18 columns of spiked urine. In Fig. 2 mass spectrometric fragmentation (MS/MS) of bovine β- casomorphin 1-4 (CM1-4, Y-P-F-P) is presented. Top trace is the synthetic standard and bottom trace the peptide isolated from urine. They are clearly the same compound.

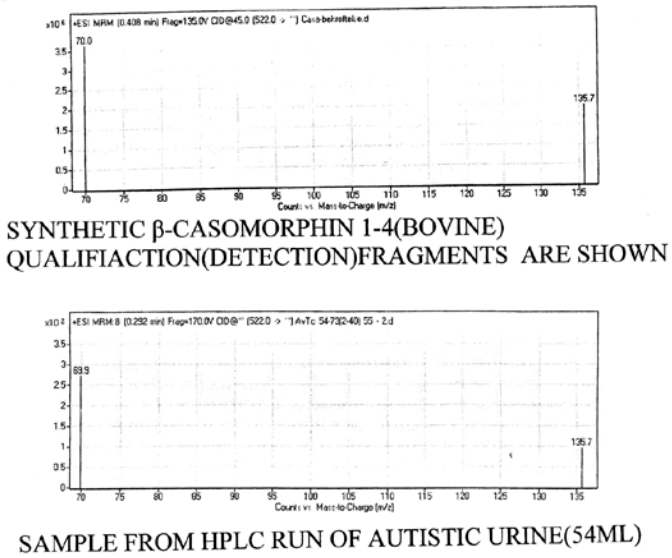
Figure 2:



Legend to Figure 2. The fragmentation pattern bovine β-casomorphin 1-4 for synthetic standard and urine derived peptide from an autistic child. The fragments are all the same and hence the two substances are equal . In B the collision energy has been decreased to keep more of the mother ion.

In Fig. 3 the reporter ions of synthetic and biological isolated CM1-4 used for routine identification is presented. The reporter peaks are picked from the dominant peaks that are characteristic for each compound examined.

Figure 3:



Legend to Figure 3. The same compound CM1-4 identified by reporter ions chosen because of size and specificity Top trace is from the commercially obtained standard, and bottom trace from an autistic child SD is ±0.4 m/z.

In Table 3 the reporter ions for glutenin derived opioids are presented. Quantification using the reporter ions is demonstrated in Fig 4. Recovery data of exorphins from spiked urine are shown in table 4.

Table 4. The percentage wise recovery of the different opioids from spiked normal urine after solid phase extraction. Number of experiments n = 6.

Peptide and Structure	Average recovery, %	RSD, %	Confidence interval (95 %)
Glumorphine A5, GYYPT	71.14	6.38	64.4 -77.8
Glumorphine A4, GYYP	80.07	11.04	68.48 -91.65
Glumorphine B4, YGGW	77.68	14.86	62.08 -93.29
Glumorphine C YPISL	82.62	6.32	75.98 -89.25
Cm1-3 YPF	90.32	4.95	85.12 -95.41
Cm1-4 YPFP	93.40	6.07	87.03 -99.77
Cm1-4amide YPFPNH2	77.05	3.27	75.61 -80.48

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CM1-5, YPFPG	91.47	7.11	84.00 -98.93
CM1-5amide, YPFPGNH2	91.83-	3.10	88.58 -95.09
CM1-7 YPFPGP	77.02	2.48	74.27 -79.50
CM1-8 YPFPGPI	90.62	4.03	86.38 -94.85

4. Discussion

According to the presented results, the exorphins were broken down in human urine, probably by peptidase(s). It is therefore critical to use a peptidase inhibitor or freezing during transport and storage of the sample material.

4.1 Degrading process in urine

The degrading process of each peptide was studied separately to prevent any of the same breakdown products from different peptides from appearing together. It is assumed that the peptide splitting enzyme is active towards the Pro-X bond because of its breakdown profile. Such an active site of the enzyme can be associated with the enzyme DPP IV (6), but could also fit post –proline splitting enzyme.

Degradation of all peptides in spiked urine was found when analyzed with the established MRM-transition (Table 2 and 3). Acetic acid is known to be a peptidase inhibitor.

4.2 Solid phase extraction

Urine and column washing solution contain a greater amount of matrix derived material, and would also be able to conceal small amounts of peptides. Recovery for each peptide can be seen in table 4 , where urine spiked with known amounts of opioids were used. Clearly, internal standards of peptides is needed for each analytical series each day. We measured the opioids in normal volunteering children from Madlavoll school who were without medicines , did not have known gastrointestinal problems or known behavioral problems. Measured opioids were found in only one of 20 normal children.

We have elsewhere shown by HPLC mass-spectrometry and MS/MS the identity of opioids in urine with known exorphins [10,11] In this paper we show how a routine screening and quantitation of the same peptides can be done . The quantitative measure is satisfactory as seen in figure 4. The importance of the opioids is evident as seen in table 5- and is reinforced by published data on gluten/casein free diet [12-18] in autistic children with demonstrated peptide and opioid increase in the urine.

Table 5. Effect of opioids on brain and behavior

Symptom	Mechanism	Reference	Test/ Comment
Hyper motoric state	Hyperdopaminergic State	19,20	Open Field
Analgesia	Opioid receptors	19	Tail flick test
Social indifference	Opioid receptors	21	Separation distress call abrogation
Hyperarousal	Dopaminergic increase	22,23	Galvanic skin test
impeded habituation	Increase in dopamine ,decrease in serotonin	22,23	Galvanic skin test
Trophic CNS effect	Interferes with cell growth and apoptosis	24,25	NMR
Stress like endocrinology	Cortisone increase	26	Dexamethasone suppression test
Nerve cell activation	Fos antigen increase	27	ELISA on brain nuclei
Hallucination Visual and auditive	Fos antigen increase in Geniculate nuclei and primary sensory cortex?	27	ELISA on brain nuclei
Anxiety	Opioids	26	Opioid receptor assay
Stereotypy	Opiate induced hyper dopaminergic state	19,20	Open field
Oxidative stress	Inhibition of cystein uptake	28	Uptake in neuroblastoma cells
Insulin release	Serum assay	29	Also glucagon release

5. Conclusions

Different exorphins must have collision energies and fragmentation voltages adjusted for optimal detection. The pre-analysis transport, storage and preparation of samples are critical to the analyses of exorphins in urine. Solid phase extraction cleaned up the sample and Citric acid or acetic acid stabilizes the exorphins so that they survive break down after melting during transport. However, citric acid is the better choice since acetic acid has a tendency to evaporate in storage from tubes used for collecting urine. As in other body fluids like CSF and blood peptidase inhibition is necessary for measuring the exorphins (31,32). The fact that mucosal damage as in celiac disorder causes demonstration of exorphins is confirmed by the same two studies (31,32).

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Conflict of interest: Dag Tveiten, Marthe Andersson and K.L Reichelt work at Lab1, NO-1337 Sandvika (next to Oslo) to develop the described assay techniques for routine tests at Lab 1. The rest of the authors are without any conflict of interest.

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