1 # Title page

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18 # Comparative distribution of somatostatin and thrittene 19 bioactive peptides in the central nervous system of rat 20 measured by radioimmunoassay

21

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38

39 ## Abstract

40 In the present paper the development and application of a novel somatostatin
41 radioimmunoassay are described. ^{125}I-labeling of Tyr$^{(0)}$-somatostain-14 (tyrosin$^{(0)}$-

42 somatostain-14) was performed by the iodogen-method and the mono-iodinated peptide
43 was separated by reversed-phase HPLC. As a practical application of the novel RIA, the
44 present work measured the somatostatin concentration parallel with the determination of
45 thrittene content in different rat brain areas. The highest somatostatin concentrations were
46 detected in the cerebellum. The thrittene immunoreactivity in the same brain parts
47 showed a similar tendency. In the presesnt study concluded that thrittene is not created
48 via a specific biosynthetical route, but from the pre-prosomatostatin.

49

50 **Keywords**

51 Thrittene, somatostatin, radioimmunoassay (RIA), iodination, central nervous system
52 (CNS)

53

54 Introduction

55 During our research we were investigating the distribution of two peptide
56 hormones from the same peptide family, namely the somatostatin and the thrittene, in the
57 rat central nervous system (CNS) with radioimmunoassay (RIA).
58 The somatostatin was discovered in 1973 by Brazeau at al. as a peptide which potently
59 inhibited growth hormone release from the anterior pituitary [1]. Originally it was
60 isolated from the hypothalamus, but thereafter was evinced in the CNS, gastrointestinal
61 tract (GIT) and the pancreas with immunohistochemistry and RIA [2, 3]. Somatostatin
62 has two biologically active form, the 28 and the 14 amino acids containing cyclic
63 polypeptides (Table 1.), which are produced in the CNS, primarily in the hypothalamic
64 neurons, furthermore in the D cells of the GIT and the pancreas [4]. From the coding
65 gene, 116 amino acids containing, preprosomatostatin is synthesized. This primary
66 precursor is cleaved to form pro-somatostatin, a further precursor consists of 92 amino
67 acids [5]. This molecule undergoes proteolytic processing at the COOH terminus as well
68 as at the HN_2 terminus. This C-terminal cleavage leads to the biologically active

69 somatostatin-28 form, from which another enzymatic cleavage results in the
70 somatostatin-14 [6-8]. From its discovery as an inhibitor, somatostatin is known to be a
71 universal inhibitory substance with multiple actions: it may act as a hormone via the
72 circulation, as a paracrine effector via local regulatory mechanisms, or as a
73 neurotransmitter via synaptic junctions [9].
74 Thrittene belongs also to the family of related peptides containing somatostatin and
75 cortistatin (Table 1.). It was identified by Ensinck at al. in 2002 and the peptide was
76 purified from monkey ileum. Its amino acid sequence, molecular mass and
77 chromatographic characteristics conformed to those of somatostatin-28 (1-13), a peptide
78 not describe heretofore. The peptide was detected in the GI tract of mammalians by
79 immunohistochemistry, and then developed a subtraction RIA method to measure the
80 peptide, and the help of this method defined the peptide concentration in different tissues
81 and proved the peptide presence in the circulation. [10]. Later confirmed, that the release
82 of the thrittene is the effect of dining and get into the circulation. The components of the
83 food affect the releasing of the peptides (somatostatin and thrittene). If the food is rich in
84 carbohydrates, it increases the concentration of the thrittene in the plasma, while the food
85 is rich in proteins that will affect the releasing of the somatostatin-28 [11]. From the fact
86 that the thrittene and the somatostatin-28 respond differently to the components of the
87 food, furthermore the histological distribution of this two peptide is diverse, concluded
88 that the synthesis of the two peptides is not related, therefore the coding gene of the
89 preprosomatostatin does not play a part in the biosynthesis of the thrittene [10, 11].
90 To confirm or confute the upper conclusion the research plane was designated to identify
91 the somatostatin and thrittene concentrations in different rat brain areas with specific RIA
92 methods, then define the somatostatin/thrittene ratio in every brain part. If this ratio
93 shows huge variation (so the ratio if this two peptide producing cells is different in the
94 investigated brain areas) we can confirm the conclusion. In that case the ratio is spreading
95 around an average, and then we need to confute the previous researches. Because this
96 could be possible in that case, when the somatostatin and the thrittene concentration
97 parallel change in different brain parts, then the ratio remains constant. But in that case
98 we need to suppose the thrittene is mostly produced during synthesis of the somatostatin
99 (but we can not exclude totally different biosynthetic pathways).

In the presented research study we investigated the distribution of two biological active peptides in the rat nervous system with radioimmunoassay. This micro analytical technique is a competitive protein binding method for the quantitative determination of antigenically active substances that are present in very small amounts, such as peptide hormones. The concentration of an unknown, non-labelled antigen is determined by comparing its inhibitory effect on the binding of radioactively labelled antigen to a specific antibody with the inhibitory effects of known standards. The used direct specific thrittene RIA method was adjusted in 2012 in our laboratory [12]. But to measure the somatostatin we educed a new method. Our aim was to develop a method, which can measure at the same time both of the biologically active forms, somatostatin-28 and 14. The only way it was possible, if in the RIA used antibodies recognize and bind to the peptide's common C-terminals.

Peptide	Sequence
somatostatin-28:	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys- Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
somatostatin-28 (1-14):	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys
somatostatin-28 (1-12):	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu
somatostatin-14:	Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
$Tyr^{(0)}$-somatostatin-14:	Tyr-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
somatostatin-14 (3-14):	Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys
thrittene:	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg
$Tyr^{(0)}$-thrittene:	$Tyr^{(0)}$-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg
cortistatin-29 (rat):	Gln-Glu-Arg-Pro-Pro-Leu-Gln-Gln-Pro-Pro-His-Arg-Asp-Lys-Lys-Pro-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys
cortistatin-14 (rat):	Pro-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys
cortistatin-29 (human):	Gln-Glu-Gly-Ala-Pro-Pro-Gln-Gln-Ser-Ala-Arg-Arg-Asp-Arg-Met-Pro-Cys-Arg-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys
cortistatin-17 (human):	Asp-Arg-Met-Pro-Cys-Arg-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys-Lys

Table 1.

Amino acid sequence of somatostatin, thrittene and cortistatin peptides.

116

117 ## Experimental

118 Materials, drugs and chemicals

119

120 Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenil glycoluril), bovine serum albumin
121 (BSA), sodium azide, glutardialdehyde, acetic acid, pentobarbitone sodium (Nembutal),
122 incomplete Freund's adjuvant, dialysis tubing and peptides (somatostatin-28,
123 somatostatin-28 (1-14), somatostatin-28 (1-12), $Tyr^{(0)}$-somatostatin-14, somatostatin-14
124 (3-14)) were bought from Sigma (St. Louis, USA). Human cortistatin-17 and cortistatin-
125 29, rat cortistatin-14 and cortistatin-29 were obtained from Bachem (Bubendorf,
126 Switzerland). Thrittene and $Tyr^{(0)}$-thrittene were synthesized by G.K. Tóth (Department
127 of Medical Chemistry, University of Szeged, Szeged, Hungary). Norit A (charcoal) and
128 dextran FP 70 were purchased from Serva (Heidelberg, Germany); carrier-free $Na^{125}I$,
129 free from reducing agent for protein iodination from Institute of Isotopes (Budapest,
130 Hungary); sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride
131 and dichloromethane from VWR International (Debrecen, Hungary); trifluoroacetic acid
132 (TFA) from Fluka (Buchs, Switzerland); HPLC-grade acetonitrile from Carlo Erba
133 (Rodano, Italy). Polypropylene RIA tubes (12 x 75 mm) were obtained from Merck
134 (Darmstadt, Germany). The animals (New Zeeland white rabbits and Wistar rats) were
135 purchased from Charles River Laboratories (Budapest, Hungary).

136

137 Description of thrittene radioimmunoassay

138

139 Thrittene RIA was performed in the study as previously described [12]. Briefly,
140 the conditions were as follows: antisera of thrittene "TH3/6" was raised against a
141 conjugate of thrittene and BSA coupled by glutaraldehyde in rabbit (dilution: 1:60000);
142 tracer: mono-^{125}I-labeled $Tyr^{(0)}$-thrittene prepared in our laboratory (3000 cpm/tube);
143 standard: synthetic thrittene ranging from 0 to 100 fmol/ml; buffer: assay prepared in 1
144 ml of 0.05 mol/l (pH 7.4) phosphate buffer containing 0.1 mol/l sodium chloride, 0.25 %

145 (w/v) BSA and 0.05 % (w/v) sodium azide; incubation: 48 h at 4 °C; separation solution:
146 charcoal/dextran/milk powder (10:1:0.2 g in 100 ml of distilled water); centrifugation:
147 4000 rpm, 4 °C, 10 min.
148
149
150 Development of somatostatin radioimmunoassay
151
152 Synthesis of somatostatin-14
153
154 Somatostatin-14: H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-
155 Cys-OH (disulfide bridge: 3-14) was prepared applying ^{t}Boc chemistry. The peptide
156 chain was elongated on a Pam resin (0.7 mmol/g) and the synthesis was carried out
157 manually. The side-chain protecting groups were the followings: Boc-Lys(2ClZ), Boc-
158 Cys(Meb) and Boc-Thr(Bzl). Couplings were performed with DCC and HOBt, without
159 difficulties. Amino acid incorporations were monitored by quantitative ninhydrin test
160 [13]. The completed peptide resin (1 g) was treated with liquid HF/dimethyl sulphide/p-
161 cresol/p-thiocresol (86:6:4:2 v/v), on 0 ºC, 45 min. HF (hydrogen fluoride) was removed
162 and the resulted free peptide was solubilized in 10 % aqueous acetic acid, filtered and
163 lyophilized. The crude peptide (150 mg) was dissolved in distilled water (100 ml) and 3
164 ml of 0.1 mmol/l potassium hexaciano ferrate (III) was added under stirring. After 3
165 hours the solution was lyophilised and purified by semipreparative RP-HPLC using a
166 solvent system of (A) 0.1 % TFA (trifluoroacetic acid) and (B) 80 % acetonitrile on a
167 Phenomenex Jupiter (Torrance, California, USA) C18 10 µm column (15 x 250 mm). The
168 HPLC apparatus was made by Knauer (Berlin, Germany). Absorbance was detected at
169 220 nm. Purity of the cyclized peptide was evaluated by analytical RP-HPLC on a 4.6 ×
170 250 mm Phenomenex Luna C18 column using a HP 1100 HPLC system made by Hewlett
171 Packard (Palo Alto, California, USA). The optimal HPLC conditions for the analytical
172 controls were: flow: 1.0 ml/min; gradient: 35-50 % in 15 min.
173
174
175 Preparation of BSA-somatostatin-14 immonogen

176

177 10 mg purified cyclized peptide (H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-
178 Phe-Thr-Ser-Cys-OH, disulfide bridge between the 3-14 Cys), 10 mg BSA and 16 µl
179 glutardialdehyde were dissolved in 4 ml of phosphate buffer (pH 7.8) at 4 °C and was
180 stirred for 12 hours. The incorporation of peptide into conjugate was monitored by
181 addition of a trace amount (10000 cpm) of ^{125}I-labelled RIA tracer to the reaction mixture
182 [39]. Dialysis was made against distilled water using dialysis tubing at 4 °C for 48 h. The
183 incorporation of label into conjugate was 73 %. The obtained conjugation mixture (BSA-
184 somatostatin-14 immunogen) was lyophilized and kept to be used for immunization.

185

186 Immunization

187

188 Approximately 800 µg BSA-somatostatin-14 conjugate was emulsified in 1 ml of
189 emulsion of Freund's complete adjuvant and sterile saline (1:1) and injected
190 intradermally, at 8-10 sites, into each of 5 young male New Zeeland white rabbits
191 (weight: 2-2.2 kg). Further immunizations with the half of starting dose of conjugate (400
192 µg/ml per animal) were made at 5-week intervals [39]. Finally, the rabbits were bled
193 from an ear vein 2 weeks after the 5th, last successive immunizations. The collected sera
194 were stored at –80 °C. The antisera were tested for titre and specificity by comparing the
195 binding of several somatostain-like peptides in RIA conditions.

196

197 Iodination of Tyr$^{(0)}$-somatostatin-14

198

199 Freshly dissolved iodogen (10 µg) in dichloromethane (100 µl) was dispersed in
200 the bottom of a polypropylene iodination vial and evaporated to dryness under nitrogen
201 flow. Subsequently, 0.25 mol/l, pH 7.4 phosphate buffer (100 µl) and Tyr$^{(0)}$-somatostatin-
202 14 (20 nmol/40 µl) were added together with 18.5 MBq/5 µl carrier-free and free from
203 reducing agent Na^{125}I. The labeling process had been allowed to run for 4 min at room
204 temperature. To terminate the iodogen mediated oxidizing reaction 0.1 % (v/v) TFA (400
205 µl) was administered into the reaction tube. Immediately thereafter, the reaction mixture
206 was loaded onto a reversed-phase HPLC column for separation.

207

208 Reversed-phase HPLC separation

209

210 Radio-labeled products of the iodogen labeling reaction were fractionated by
211 means of reversed-phase HPLC technique. The HPLC system comprised a Merck-Hitachi
212 pump (type: L-7100), a Rheodyne injector (type: 7161), a LiChrospher 100 RP-18 (5 µm,
213 4 x 250 mm) reversed-phase column and a Merck fraction collector (type: L-7650). The
214 column was washed with acetonitrile (solvent B), then equilibrated with 0.1 % (v/v) TFA
215 (solvent A) prior to use. HPLC-grade acetonitrile as an organic component along with
216 aqueous solution of 0.1 % (v/v) TFA was employed to elute the radio-labeled peptides
217 and free $^{125}I^-$ ion at a flow rate of 0.5 ml/min. Fractions at a volume of 0.5 ml were
218 collected. Finally, the radioactivity of an aliquot volume (5 µl) of each fraction was
219 determined in a gamma counter (Gamma NZ 310).

220 Identification of various radioactive products

221

222 Free iodine and mono- or di-iodinated forms of Tyr$^{(0)}$-somatostatin-14 occurred as
223 three distinct peaks after reversed-phase HPLC separation. Identification of the various
224 products based on their order of appearance (free iodine occurring in the first peak) or
225 binding to the raised somatostatin (SOM4/5) antibody (mono- or di-iodinated forms).
226 Subsequently, determination of specific radioactivity of the radio-labeled peptides was
227 performed by the self-displacement method described by Morris [14] to distinguish the
228 mono-iodinated from the di-iodinated one.

229

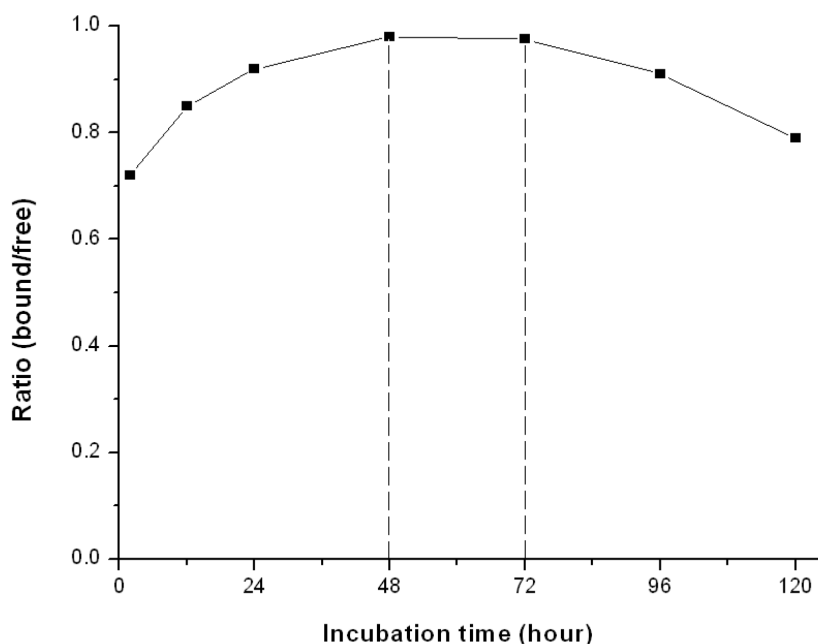
230 Description of somatostatin radioimmunoassay

231

232 *Antiserum:* SOM4/5 was raised against a conjugate of somatostatin-14 and BSA
233 coupled by glutaraldehyde in a rabbit.
234 *Tracer:* mono-^{125}I-labeled Tyr$^{(0)}$-somatostatin-14 was prepared in our laboratory.
235 *Standard:* somatostatin-14 was used as a RIA standard ranging from 0 to 200
236 fmol/ml.

237 *Buffer:* the assay was prepared in 1 ml 0.05 mol/l (pH 7.4) phosphate buffer
238 containing 0.1 mol/l sodium-chloride, 0.25 % (w/v) BSA and 0.05 % (w/v) sodium azide.
239 *Assay procedure:* 100 µl antiserum (working dilution 1:3000), 100 µl RIA tracer
240 (3,000 cpm/tube) and 100 µl somatostatin-14 standard or unknown samples were
241 measured into polypropylene tubes with the assay buffer. After 48 h incubation at 4 °C,
242 the antibody-bound peptide was separated from the free one by addition of 100 µl
243 separating solution (10 g charcoal, 1 g dextran and 0.2 g commercial fat-free milk powder
244 in 100 ml distilled water) [39]. Following centrifugation (4000 rpm, 4 °C, 10 min) the
245 tubes were gently decanted and the radioactivity of the precipitates was measured in a
246 gamma counter (Gamma NZ310). Somatostatin-14 concentrations of the unknown
247 samples were read from a calibration curve.
248 Every step of the RIA determination (preparation, incubation, separation) was carried out
249 at low temperature, on ice or at 4 °C. The optimal incubation time for this somatostatin
250 RIA is 48-72 hours at 4 °C (Fig. 1).

251



252

253 **Fig. 1.**

254 The effect of incubation time on binding of somatostatin RIA tracer (mono-^{125}I-labeled
255 Tyr$^{(0)}$-somatostatin-14) to SOM4/5 antibody at 4 °C.

256

257 Animal tissue extraction

258

259 Overnight fasting male Wistar rats (250-300 g) were anaesthetized with 50 mg/kg
260 i.p. pentobarbitone sodium (Nembutal), killed by cervical dislocation and exsanguinated.
261 Thereafter, the whole brain was removed and the following five brain areas were
262 separated and collected: telencephalon, mesencephalon, cerebellum, hypothalamus and
263 medulla oblongata. The tissue samples were placed on ice, after cooling, cleaned,
264 weighed and homogenized in ice-cold distilled water to give a 10 % (w/v) homogenate.
265 Finally the homogenates were centrifuged (10000 rpm, 4 °C, 30 min) and the
266 supernatants were transferred for somatostatin and thrittene radioimmunoassay
267 measurements. Content of peptides of various brain areas were expressed as fmol/mg wet
268 tissue weight. Data are expressed as means ± standard error of means (SEM).

269

270 **Ethics**

271

272 The experimental protocols applied in the present work conformed to the
273 European Community guiding principles for the care and use of laboratory animals and
274 were approved by the Local Ethical Committee of the University of Debrecen, Hungary.

275

276 Results

277 Synthesis of somatostatin-14

278

279 The lyophylisation of the pooled fractions led to 38.1 mg of pure somtostain-14,
280 resulting 25.4 % overall yield. Analytical RP-HPLC: Phenomenex Luna (4.6 x 250 mm)

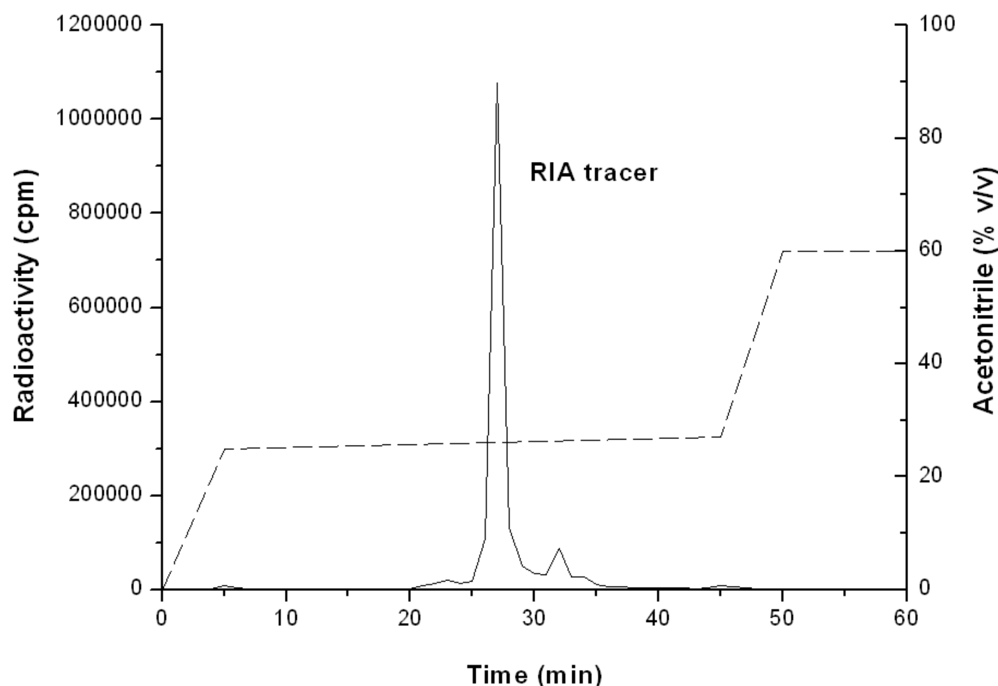
281 C18 column; flow: 1.0 ml/min; acetonitrile gradient: 35-50 % in 15 min; retention time:
282 7.18 min; mass spectrometry: measured molecular weigh: 1637.4, calculated: 1637.89.
283 (The synthetic peptide was characterized by mass spectrometry using a Finnigan TSQ
284 7000 tandem quadrupol mass spectrometer equipped with electrospray ion source.) The
285 pure peptide was used as RIA standard and for immunization in conjugated form.

286

287 Reversed-phase HPLC separation of iodinated $Tyr^{(0)}$-somatostatin-14 forms

288

289 On the basis of radioactivity measurements of the fractions collected, three
290 distinct peaks occurred with respective retention times (Rt). The first one represented
291 radioactivity of the free $^{125}I^{-}$ ion (Rt: 5 min), the second peak (Rt: 27 min) showed
292 radioactivity of the mono-iodinated form of $Tyr^{(0)}$-somatostatin-14, whereas the third
293 peak represented the di-iodinated form of $Tyr^{(0)}$-somatostatin-14 (Rt: 32 min). Specific
294 radioactivity values of mono- and di-iodinated peptides were 71.4 and 146.6 TBq/mmol.
295 Radioactivity of the $Tyr^{(0)}$-somatostatin-14 bound iodine accounted for approximately 84
296 % (mono-iodinated 74 %, di-iodinated 10 %) of the total amount of activity consumed.
297 Radioactivity of unincorporated $^{125}I^{-}$ ion (Rt: 5 min) was 6 %. The appropriate gradient of
298 acetonitrile to separate mono- and di-iodinated forms of $Tyr^{(0)}$-somatostatin-14 ranged
299 from 25 to 27 % over 40 min (Fig. 2).



300

301 **Fig. 2.**

302 Radiochromatogram of the Tyr$^{(0)}$-somatostatin-14 forms separated by reversed-phase
303 HPLC. The dashed line shows the acetonitrile gradient, the continuous line the
304 radioactivity of fractions collected.

305

306 Characterization of antiserums

307

308 The raised somatostatin antiserums were tested for titre and specificity by
309 comparing the binding of several somatostatin-like peptides.
310 The titre values of the somatostatin antiserums were determined in assay conditions. The
311 titre dilutions were as follows: SOM1/5 1:8000; SOM2/5 1:17000; SOM3/5 1:5000;
312 SOM4/5 1:30000; SOM5/5 1:23000. Antiserum (SOM4/5) with the highest titre proved
313 to be most successful for the development of RIA. This somatostatin antiserum, raised
314 against a conjugate of BSA and somatostatin-14 turned to be C-terminal specific based
315 on cross-reaction studies (Table 2).

316

317

Peptides	Cross-reactivity (%)
Somatostatin-14	100.00
Tyr$^{(0)}$-somatostatin-14	95.54
Somatostatin-14(3-14)	91.32
Somatostatin-28	86.27
Somatostatin-28(1-14)	0.00
Somatostatin-28(1-12)	0.00
Costistatin-14 (rat)	11.67
Cortistatin-29 (rat)	6.56
Cortistatin-17 (human)	7.47
Cortistatin-29 (human)	5.20
Thrittene	0.00
Tyr$^{(0)}$-thrittene	0.00

318–330

331 **Table 2.**

332 Cross-reactivity data of the SOM4/5 somatostatin antiserum. The ID_{50} of somatostatin-
333 14 calibration curve served as a reference value.

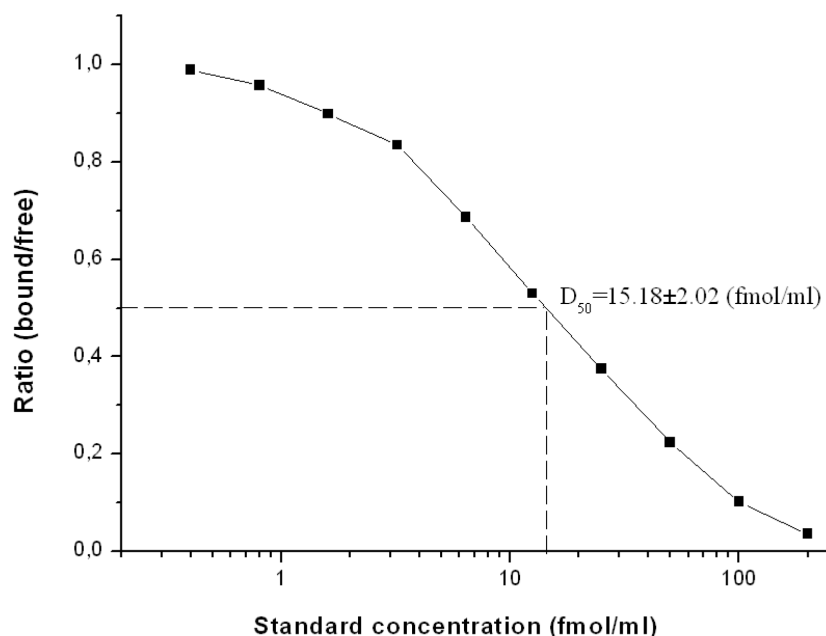
334

335 Somatostatin radioimmunassay characteristics

336

337 The average ID_{50} value (i.e. the concentration of RIA standard (somatostatin-14),
338 that displaces 50 % of the tracer from the antibody) was 15.18±2.02 fmol/ml as
339 determined in ten consecutive assays (Fig. 3).

340



341

342 **Fig. 3.**

343 Calibration curve of the somatostatin radioimmunoassay.

344 *Sensitivity*

345 Sensitivity of an assay is the minimum detectable concentration (detection limit)
346 of the antigen. The detection limit of our somatostatin RIA was calculated from a
347 decrease in binding of two standard deviations in the mean of zero standard of ten
348 replicates [40]. This value was 0.24 fmol/ml. The lowest level of somatostatin-14 that can
349 be detected by this assay is approximately 0.2 fmol/ml.

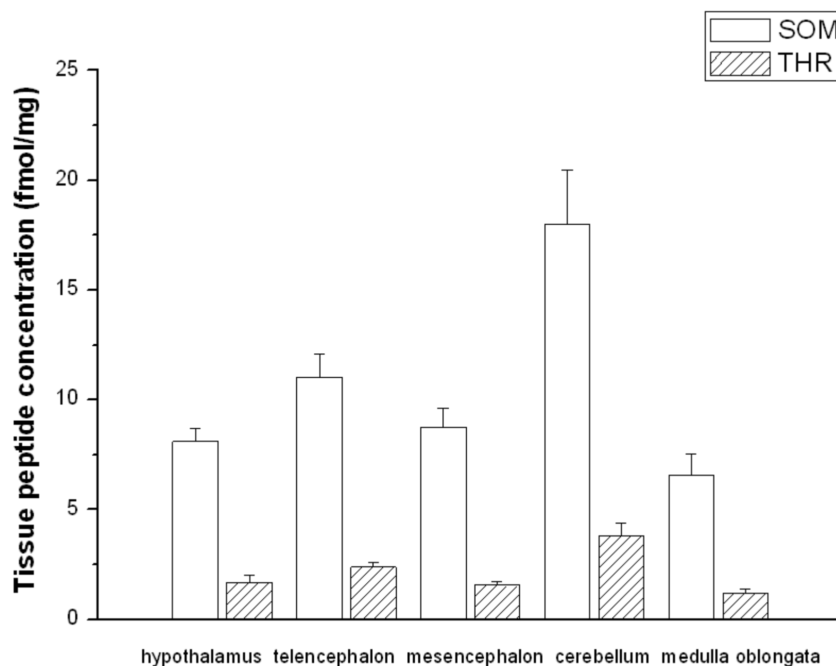
350 *Precission*

351 Intra-assay precission (within assay variation) and inter-assay precission (between
352 assay variation) was performed on three pooled samples containing varying
353 concentrations (low, medium, high) of somatostatin-14. The measured concentrations of
354 somatostatin-14 of the samples and the calculated data are presented in Table 3. Shown
355 data are from ten duplicate determinations for within assay variation and ten consecutive
356 duplicate determination for between assay variation.

357

Sample	Intra-assay		Inter-assay	
	mean (fmol/ml)	CV (%)	mean (fmol/ml)	CV (%)
low	4.2	8.3	4.8	10.5
medium	12.4	7.0	13.1	9.9
high	20.4	6.5	19.3	8.3

358
359 **Table 3.**
360 Values of intra-assay and inter-assay coefficient of variation
361
362 Somatostatin and thrittene contents of brain samples
363
364 Somatostatin immunoreactivity was present in the examined brain areas of rat
365 with highest concentrations in the cerebellum followed by telencephalon, mesencephalon,
366 hypothalamus and medulla oblongata. The thrittene immunoreactivity in the different
367 brain parts showed a very similar tendency. The highest thrittene concentration was
368 measured in the cerebellum, lowest in the medulla oblongata. The somatostatin
369 concentration in every investigated tissue sample was approximately 5-fold higher than
370 the determined thrittene content in the same brain area (Fig. 4).



371

372 **Fig. 4.**

373 Distribution of somatostatin and thrittene in various rat brain areas measured by
374 radioimmunoassay. Somatostatin and thrittene contents are expressed as fmol/mg wet
375 tissue weight. Data are presented as means ± S.E.M. Abbreviations: SOM – somatostatin,
376 THR – thrittene

377

378 Discussion

379

380 The present study provieded a descriptoin of a highly sensitive and specific
381 radioimmunoassay method to measure a peptide called somatostatin in the radio-
382 analytical laboratory of our department. The objective of the present work was to develop
383 a method, which can measure both of the biologically active forms of somatostatin
384 (somatostatin-28 and 14) in the biological samples. The only way it was possible, if in the
385 RIA used antibodies recognize and bind to the common C-terminals of the peptides. For
386 this purpose glutaraldehyde coupling was used in this study to create the immunogen, due
387 to the fact that this fixation method crosses the peptide through the N-terminal end to the
388 carrier peptide (BSA). Consequently, the C-terminal part of the peptide remained free so
389 the generated antibodies (mainly IgG) were produced against this sequence hence the

390 antibody recognizes the common C-terminal region of somatostatin-28 and 14. In the
391 cross-reaction examination of our new "SOM4/5" antiserum we found a limited binding
392 (5-10 %) to the rat and human cortistatin peptides (Table 1) but the values of these
393 bindings are not important, so the antibody recognizes and binds mainly somatostatin-28
394 and 14.
395 ^{125}I is the most commonly used isotope for labeling of peptide hormones in order to
396 perform RIA measurements. The widely applied oxidizing agents are: chloramine-T [15-
397 23, 40], hydrogen peroxide [24] and iodogen [12, 16, 25-31, 39]. After the labeling
398 procedure the mono-iodinated Tyr$^{(0)}$-somatostatin-14, serving later as RIA tracer, must be
399 separated from the other compounds (di-iodinated Tyr$^{(0)}$-somatostatin-14, unlabelled
400 peptide, unincorporated iodine, oxidatively damaged labeled or unlabelled products). The
401 present work provides a description of iodination, using iodogen, a solid-phase oxidizing
402 agent, and separation by reversed-phase HPLC of Tyr$^{(0)}$-somatostatin-14. High purity of
403 the mono-^{125}I-labelled Tyr$^{(0)}$-somatostain-14, as a RIA tracer, is of crucial importance to
404 carry out reliable measurements. Accordingly, the purpose of various iodination and
405 separation procedures (gel filtration [16, 21, 23, 30, 32, 40], starch gel electrophoresis
406 [33], paper electrophoresis [15, 20], thin layer chromatography [25, 28], ion-exchange
407 chromatography [19, 34], reversed-phase HPLC [12, 15, 17, 22, 29, 31, 35-37, 39]) is to
408 achieve the highest yield and purity of mono-iodinated form. Under optimal labeling and
409 purification conditions as described above, the fraction of the mono-iodinated form can
410 be as high as 70-75 %. The specific radioactivity values of the mono- and di-iodinated
411 forms of Tyr$^{(0)}$-somatostatin-14 are nearly identical with the theoretical values (78.63 and
412 157.26 TBq/mmol) [38], documenting high efficacy of the reversed-phase HPLC
413 separation technique employed. To prepare the optimal RIA tracer, the radioactive
414 labeling must be as far as possible from the binding region. This problem was solved by
415 coupling a Tyr amino acid, which is essential for the iodination, in zero position to the N-
416 terminal region of somatostatin-14. Incorporation of iodine into a peptide can modify the
417 structure of the molecule, and if this modification is at the binding region, it can change
418 the binding property or can extinguish it. In our case there was no such problem as the C-
419 terminal part is recognized by the antibody, the radioactive labeling was performed at the
420 zero position of the N-terminal region.

421 As a practical application of the novel RIA, in this study the somatostatin concentration
422 was measured parallel with the determination of thrittene content in different rat brain
423 areas. The highest somatostatin concentrations were detected in the cerebellum followed
424 by telencephalon, mesencephalon, hypothalamus and medulla oblongata. The thrittene
425 immunoreactivity in the same brain parts showed a similar tendency. It is striking that in
426 all cases the ratio of the two peptides are almost the same, namely the amount of the
427 somatostatin measured in the same samples is about 5 times the thrittene concentration.
428 (Our work group has found similar results in a previous project, when comparing the
429 somatostatin and thrittene concentration of specific intestinal regions.) These results
430 verify our following theory: thrittene is not created via a specific biosynthetical route, but
431 from the pre-prosomatostatin, and is one of its split products. If it was created in a
432 different way, then in our opinion at least in some organs the ratio of the two peptides
433 would be different. In our future experiments concerning digesting we will try to exactly
434 prove (or peremptorily rule out) that thrittene is the degradation product of the S-28.
435 According to our hypothesis the peptide is made when the 28-amino acid somatostatin
436 splits, and from the C terminal region the S-14 forms (also with full biological activity).
437 The remaining N terminal region contains the structure of the thrittene, however, one
438 additional amino acid (Lys) has to be split off beforehand. During the experiment we will
439 incubate native somatostatin-28 at 37°C in homogenisation of different organs for
440 different amounts of time, giving the peptidase enzymes opportunity to split the S-28, and
441 the peptides created during this process. After this, as the first step, using specific RIA
442 methods, we will measure the thrittene and somatostatin concentrations of the organ
443 media. If the thrittene amount in the incubated samples exceeds the basic amount, or if
444 this shows a growing tendency during the incubation time, this will be an evidence for the
445 biosynthesis of the thrittene. Of course, the peptides created during the incubation will be
446 analyzed and identified. Methods for these may be HPLC separation and then amino acid
447 analysis.

448

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