

Profiling of Adrenocorticotrophic Hormone and Arginine Vasopressin in Human Pituitary Gland and Tumor Thin Tissue Sections using Droplet-Based Liquid Microjunction Surface Sampling-HPLC-ESI-MS/MS

Vilmos Kertesz^{*1}, David Calligaris², Daniel R. Feldman², Armen Changelian², Edward R. Laws², Sandro Santagata³, Nathalie Y.R. Agar^{*2,4} and Gary J. Van Berkel¹

¹ Organic and Biological Mass Spectrometry Group
Chemical Sciences Division, Oak Ridge National Laboratory
Oak Ridge, TN 37831-6131

² Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115-6110

³Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115-6110

⁴ Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115-6110

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21 *Corresponding authors: 25
22 VK: 865-574-3469 26 NYRA: 617-525-7374
23 Fax: 865-576-8559 27 Fax: 617-264-6316
24 Email: kerteszv@ornl.gov 28 Email: Nathalie_Agar@dfci.harvard.edu

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ABSTRACT

43 Described here are the results from the profiling of the proteins arginine
44 vasopressin (AVP) and adrenocorticotropic hormone (ACTH) from normal human
45 pituitary gland and pituitary adenoma tissue sections using a fully automated droplet-
46 based liquid microjunction surface sampling-HPLC-ESI-MS/MS system for spatially
47 resolved sampling, HPLC separation, and mass spectral detection.

48 Excellent correlation was found between the protein distribution data obtained
49 with this droplet-based liquid microjunction surface sampling-HPLC-ESI-MS/MS system
50 and those data obtained with matrix assisted laser desorption ionization (MALDI)
51 chemical imaging analyses of serial sections of the same tissue. The protein
52 distributions correlated with the visible anatomic pattern of the pituitary gland. AVP was
53 most abundant in the posterior pituitary gland region (neurohypophysis) and ACTH was
54 dominant in the anterior pituitary gland region (adenohypophysis).

55 The relative amounts of AVP and ACTH sampled from a series of ACTH
56 secreting and non-secreting pituitary adenomas correlated with histopathological
57 evaluation. ACTH was readily detected at significantly higher levels in regions of ACTH
58 secreting adenomas and in normal anterior adenohypophysis compared to non-
59 secreting adenoma and neurohypophysis. AVP was mostly detected in normal
60 neurohypophysis as anticipated.

61 This work demonstrates that a fully automated droplet-based liquid microjunction
62 surface sampling system coupled to HPLC-ESI-MS/MS can be readily used for spatially
63 resolved sampling, separation, detection, and semi-quantitation of physiologically-
64 relevant peptide and protein hormones, such as AVP and ACTH, directly from human

65 tissue. In addition, the relative simplicity, rapidity and specificity of the current
66 methodology support the potential of this basic technology with further advancement for
67 assisting surgical decision-making.

68

69 **Keywords:** liquid microjunction, droplet-based liquid extraction, autosampler,
70 spatial distribution, human pituitary, protein, adrenocorticotrophic hormone (ACTH), AVP
71 (vasopressin), pituitary adenoma.

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74 **INTRODUCTION**

75 During surgery, preliminary diagnosis of tissue biopsies is currently rendered by
76 trained pathologists via the microscopic review of hematoxylin and eosin (H&E) stained
77 thin tissue sections of the aforementioned biopsy samples. This process including the
78 cutting and mounting of the tissue typically takes about 20-30 minutes [1,2]. A more
79 detailed evaluation, which usually involves the characterization of the expression levels
80 of important diagnostic molecules, such as proteins, is not performed in an
81 intraoperative setting [3,4,5]. The customary method used for the detection of selected
82 protein biomarkers is immunohistochemistry (IHC), which relies on recognition of protein
83 antigens by antibodies that are generated against relevant epitopes of the biomarker of
84 interest [6]. Although IHC can reveal the presence of a protein biomarker with high
85 spatial resolution, it is time consuming, and limited by the quality and specificity of the
86 antibody. Moreover, the simultaneous characterization of multiple proteins is generally
87 impractical in the clinical setting. Hence, final diagnostic reports take days to issue and
88 significant amounts of information of potential importance to clinical care are left
89 unanalyzed within the tissue, and not available at the time of surgical resection to guide
90 decision-making.

91 Recently, significant strides have been made in the development of platforms
92 that can provide near real-time molecular information in the intraoperative setting. For
93 example, ambient ionization approaches such as desorption electrospray ionization
94 mass spectrometry (DESI MS) [1,7,8,9] and rapid evaporative ionization mass
95 spectrometry (REIMS) [2,10] have been used to monitor the lipids and metabolites
96 within tissue biopsy specimens. This information can be used to classify tumors, to

97 provide important prognostic information such as tumor subtype and grade and to
98 evaluate the molecular margins at the outer limits of a surgical resection thereby
99 informing the surgeon if the tissue is free of tumor cells. For example, a DESI MS
100 molecular profile can be typically acquired from a single sample location within 2
101 minutes after mounting [1] or along a line from a tissue on a slide (smear or swab) in
102 several minutes. A more complete two-dimensional molecular image can be acquired
103 from a mounted tissue section in about an hour [1,11], that can be used to correlate
104 underlying histology with the presence of molecular signatures. Single point profiles are
105 appropriate for rapid data acquisition during a surgical procedure while 2D molecular
106 imaging is done in the research setting to carefully validate signatures relative to classic
107 histopathology criteria [1,9]. REIMS has also shown potential for near-real-time
108 characterization of human tissue *in vivo* by analysis of the aerosol released during
109 electrosurgical dissection [2]. Based on (mostly) lipidomic profiles acquired, the REIMS
110 approach provided accurate distinction between histological and histopathological tissue
111 types.

112 Despite the ability of both DESI and REIMS to provide near real-time molecular
113 information from the tissue at stake, both are still limited mainly to the analysis of lower
114 molecular weight biomolecules such as metabolites, fatty acids, and lipids. The ability
115 to characterize quickly the tissue distribution of larger macromolecular biomarkers like
116 peptides and protein would harness the diagnostic value of validated IHC approaches
117 for surgical decision-making.

118 Direct liquid extraction based surface sampling/ionization probe technology
119 coupled with mass spectrometry [12,13,14,15,16] is one alternative approach that has

120 been proven capable of sampling and analyzing proteins from biological samples
121 [17,18,19,20,21,22,23]. Commercial systems like the Liquid Extraction Surface Analysis
122 (LESA) system from Advion have been used for extraction and direct nanoESI-MS
123 analysis of hemoglobin variants and other proteins from blood spot [17,20,21,22] and
124 bacterial colonies [18,23]. However, because these approaches employ simple
125 extraction and follow-up direct ESI-MS analysis the possibility of varying matrix effects
126 masking the targeted biomarkers of interest is a potential concern [24]. The use of
127 HPLC coupled with ESI-MS/MS for detection of targeted proteins facilitates the
128 evaluation of complex sample matrices, enables differentiation between the particular
129 disease related materials and enables absolute analyte quantitation.[25] However, this
130 approach traditionally requires extensive sampling and processing [26] or
131 homogenization of tissues [27], followed by extraction and cleanup steps. While an
132 unattended workflow might be employed for this multi-step process using conventional
133 laboratory preparative schemes and robots, these time-consuming steps render the use
134 of traditional HPLC/MS unrealistic for real-time or near real-time diagnostic applications.
135 However, the challenge of providing spatially resolved molecular information for
136 peptides or proteins in a time frame currently relevant for intraoperative work (e.g., <10
137 minutes) could currently be effectively addressed using liquid extraction-based surface
138 sampling coupled with high performance liquid chromatography (HPLC) electrospray
139 ionization-tandem MS (ESI-MS/MS). Such systems have been successfully used for
140 spatially resolved sampling and detection of drugs and metabolites from animal tissue
141 sections [28,29,30,31,32,33,34] and proteins from dried blood spots [30]. The use of
142 the liquid-extraction based sampling probes provides a simple and quick means to

143 acquire a representative sample that can be immediately injected on column for
144 analysis. Sampling and analyses can be completed in less than 10 minutes
145 [28,29,30,31,32,35].

146 We here report proof-of-principle data demonstrating that a fully automated
147 droplet-based liquid microjunction surface sampling probe-HPLC-ESI-MS/MS system
148 can be used for spatially resolved sampling, HPLC separation, and mass spectral
149 detection of adrenocorticotrophic hormone (ACTH) and arginine vasopressin (AVP) from
150 normal human pituitary gland and ACTH secreting and non-secreting human pituitary
151 adenomas. Sampling and analysis were completed in about 10 minutes per spot
152 sampled. Excellent correlation was found between the protein distribution data obtained
153 with this system and the data from matrix assisted laser desorption ionization (MALDI)
154 chemical imaging analyses of serial sections of the same tissue. The analysis of ACTH
155 and AVP levels in ACTH secreting and non-secreting pituitary adenomas also
156 corroborated results from histopathology segmentation of the tumor tissue sections. In
157 addition, the relative simplicity, rapidity and specificity of the current methodology
158 support the potential of this basic technology, with further advancement, for assisting
159 surgical decision-making.

160

161

162 **EXPERIMENTAL SECTION**

163 **Chemicals.** LC-MS grade Chromasolv[®] solvents 100/0.1 (v/v) water/formic acid
164 (FA) and 100/0.1 (v/v) acetonitrile (ACN)/FA were obtained from Sigma Aldrich (St.
165 Louis, MO, USA). All other solvents used in this study were of HPLC quality and

166 purchased from Sigma Aldrich. AVP and ACTH standard (compounds **1** and **2** in
167 Scheme 1, respectively), sinapinic acid (SA) and trifluoroacetic acid (TFA) were
168 obtained from Sigma Aldrich.

169 **Pituitary Tissue Preparation.** All research subjects were recruited from surgical
170 candidates at the neurosurgery clinic of the BWH, and gave written informed consent to
171 the Partners Healthcare Institutional Review Board (IRB) Protocols. Pituitary adenoma
172 samples were obtained from the BWH Neuro-Oncology Program Tissue Committee,
173 and analyzed under approved IRB protocol. Pituitary adenoma samples were originally
174 diagnosed as ACTH secreting pituitary adenoma (A3, A4, and A5) and non-secreting
175 adenoma (N1) based on the clinical findings. The normal pituitary tissue used in these
176 experiments came from a 48-year-old female who passed away from a
177 leiomyosarcoma. The post-mortem interval until the autopsy was conducted was 18
178 hours. Nothing from the autopsy report indicated that there was anything abnormal
179 about this specific tissue. Tissue sections were prepared on a Microm HM 550 (Thermo
180 Scientific, Waltham, MA, USA) with the microtome chamber chilled at -22 °C and the
181 specimen holder set at -18 °C. The sections were prepared at a thickness of 10 µm for
182 MALDI chemical imaging and histochemistry analysis, and 40 µm for liquid extraction-
183 HPLC-ESI-MS/MS analysis. For MALDI chemical imaging, the sections were thaw
184 mounted onto indium tin oxide (ITO) glass slides (Bruker Daltonics, Billerica, MA). For
185 liquid extraction-HPLC-ESI-MS/MS and histochemistry analyses, the sections were
186 mounted on 1" x 3" optical slides (Fisher, Pittsburgh PA). The sections were kept at -80
187 °C until analysis. Before analysis they were brought to room temperature over 30
188 minutes inside a desiccator. For each mass spectrometry analysis, lipids were removed

189 by washing each tissue with 70% EtOH for 2 minutes, followed by fresh 70% EtOH for 1
190 minute, and then 95% EtOH for 1 minute. After this washing, the slides were dried in a
191 desiccator for at least 30 minutes.

192 **Hematoxylin and Eosin Staining for Histochemical Analysis.** The
193 histochemical analysis was performed on a sister section of those samples that were
194 analyzed by mass spectrometric techniques. The protocol was done as previously
195 described. [36] Sections were then dried at room temperature in a hood and covered
196 with histological mounting medium (Permount®, Fisher Chemicals, Fair Lawn, NJ)
197 followed by a glass cover slide.

198 **MALDI Chemical Imaging.** The normal pituitary section was prepared by
199 depositing matrix containing sinapinic acid (10 mg/mL in 60/40/0.1 (v/v/v)
200 ACN/water/TFA) onto each ITO slide using an ImagePrep automated sprayer (Bruker
201 Daltonics, Billerica, MA, USA). MALDI chemical imaging was then performed using an
202 UltrafleXtreme MALDI TOF/TOF (Bruker Daltonics, Germany) equipped with a 1 kHz
203 smartbeam laser. The spectra were acquired in positive linear mode with a mass range
204 of *m/z* 500-25,000 using an external mass calibration based on a mixture of peptide and
205 protein calibration standards. Each pixel in the chemical image was acquired with 800
206 laser shots and the chemical image was acquired with 100 µm spatial resolution. The
207 MALDI images were created with the FlexImaging 4.0 software package (Bruker
208 Daltonics, MA) using a root mean square normalization method.

209 **Liquid Microjunction Surface Sampling-HPLC-ESI-MS/MS Analysis.** High
210 contrast/resolution optical images of tissue samples mounted on glass slides 1" x 3" in
211 size were acquired individually pre-analysis using a Dino-Lite Premier digital

212 microscope (AnMo Electronics Corp., Taiwan) and associated software at a resolution
213 of 3150 dpi (about 40x zoom; samples A3, A4, A5 and N1) and 1455 dpi (about 18.5x
214 zoom; samples Pit6) for best visualization of morphological details. For analysis, glass
215 slides holding the tissue sections were secured in a custom sample holder of size equal
216 to a microtiter plate (Figure 1a). The custom sample holder was designed in
217 OpenSCAD [37] and printed using acrylonitrile butadiene styrene (ABS) plastic
218 employing a Dimension 1200es (Stratasys, Eden Prairie, MN) 3D printer. The holder
219 included a spring-loaded sample region, space for 4 individual extraction solvent vials
220 and a smaller diameter hole used in locking the needle guide to assist with switching the
221 injector needle configuration into surface sampling mode. The spring-loaded
222 mechanism was an improvement over the last series of our 3D printed custom sample
223 trays [32] enabling a quicker sample placement and removal as compared to using
224 double sided tape to secure the sample to the holder. The optical image of the mounted
225 tissue for selection of surface locations to be sampled was acquired using a flatbed
226 scanner (model Perfection v300 Photo, Epson America Inc., Long Beach, CA, USA)
227 controlled with the in-house developed software dropletProbe Premium[©]. The surface
228 sampling process utilized an HTS-PAL autosampler (LEAP Technologies Inc., Carrboro,
229 NC) equipped with a 100- μ L L-MARK[®] 22s-gauge gastight syringe with a fixed needle
230 (part no: LMK- 2620719, 152 μ m i.d., 717 μ m o.d., LEAP Technologies Inc.). This setup
231 was similar to that described previously [28,29,31,32]. Changes included the use of a
232 spring loaded custom 3D printed sample holder (see above) and entire control of the
233 autosampler by dropletProbe Premium[©] rather than by autosampler software LEAPShell
234 3TM (LEAP Technologies Inc.) [28,29,32] or by mass spectrometry software AnalystTM

235 (AB SCIEX, Concord, Ontario, Canada) [31]. This latter change shortened the time for
236 method development and provided more precise positioning of the autosampler syringe
237 needle along the vertical Z-axis for an individual surface location by making a needle to
238 surface distance measurement just prior to the sampling process. (see detailed
239 discussion below) A standard HPLC/MS hardware profile in AnalystTM was responsible
240 for HPLC and MS control, and data acquisition. Communication control and data flow
241 between the instruments are illustrated in Scheme 2.

242 Surface locations to be sampled were selected using the optical image. Once
243 the sample queue was completed (Supplemental Figure S1), the required HPLC/MS
244 acquisition method and MS data file names were transferred into AnalystTM via a text file
245 to create an AnalystTM sample queue. With the system controlled by dropletProbe
246 Premium[®], surface analysis was commenced by the autosampler first drawing 5 μ L of
247 90/10/0.1 (v/v/v) water/ACN/FA extraction solvent from a vial in one of the built-in vial
248 holders (Figure 1a) located on the custom sample tray into the syringe needle and
249 barrel. This was followed by switching the syringe needle configuration into surface
250 sampling mode using the needle lock hole (Figure 1a) also located on the custom
251 sample tray. After this, the laser distance unit was positioned above a surface location
252 to be sampled where the laser sensor-to-surface distance (D) was measured (Figure
253 1d, I). Using D and a previously determined vertical offset (D_f) between the laser sensor
254 and the probe (tip of the autosampler syringe needle), the actual probe-to-surface
255 distance ($D_z = D - D_f$) was calculated in dropletProbe Premium[®]. The syringe needle
256 was then positioned above the given surface spot and the probe was moved down
257 vertically by $D_z - d_0$ to obtain the predetermined optimal $d_0=0.2$ mm probe-to-surface

258 distance (Figure 1d, II). As illustrated in Figure 1d (III), this step was followed by
259 dispensing a discrete volume of extraction solvent (2 μ L in this work) onto a surface
260 creating a liquid junction between the needle and the surface (Figures 1b and 1c). After
261 a 2 s extraction time (Figure 1d, IV), the liquid was drawn back into the syringe needle
262 (Figure 1d, V), followed by switching the needle configuration from surface sampling
263 mode to injection mode (needle leveled with the needle guide), and the extract
264 transferred to an injector and injected onto a XBridgeTM BEH300 C4 column (2.1 x 100
265 mm, 3.5 μ m particle size; Waters, Milford, MA, USA) for subsequent HPLC/MS analysis
266 employing an Agilent 1100 HPLC pump (Agilent Technologies, Santa Clara, CA) to
267 deliver the separation solvents coupled to an API 4000 triple quadrupole mass
268 spectrometer (AB SCIEX, Concord, Ontario, Canada). HPLC separation solvents A and
269 B were 100/0.1 (v/v) water/FA and 100/0.1 (v/v) ACN/FA, respectively. The 10-min-long
270 gradient separation included the following steps: 0-0.5 min: 90% A; 0.5-3 min: linear
271 gradient to 70% A; 3-5 min: linear gradient to 20% A; 5-6 min: linear gradient to 90% A;
272 and 6-10 min: 90% A. Solution flow rate was 200 μ L/min.

273 Selected reaction monitoring (SRM) transitions of m/z 543.2 \rightarrow 328.3 and m/z
274 757.8 \rightarrow 876.2 were monitored for AVP (cpd **1**) and ACTH (cpd **2**), respectively using
275 positive ion mode ESI with an emitter voltage of 5.0 kV employing collision energies of
276 27 eV (cpd **1**) and 23 eV (cpd **2**) with a declustering potential of 80 V. Dwell time was
277 50 ms for each transition monitored. Turbo sprayer heater temperature was 200 °C.
278 Mass spectrometry conditions were optimized infusing 0.2 μ M solutions of the
279 respective proteins in 50/50/0.1 (v/v/v) water/ACN/FA. Scheme 1 shows the compound
280 structures and the monitored precursor ions.

281

282 **RESULTS AND DISCUSSION**

283

284 *Profiling Normal Cadaveric Human Pituitary Gland Tissue.* In order to assess the
285 suitability of the current liquid microjunction surface sampling-HPLC-ESI-MS/MS system
286 towards protein detection sister sections of a non-diseased cadaveric human pituitary
287 gland (coded Pit6) were analyzed by MALDI imaging and by the aforementioned setup.
288 Figure 2a shows an optical image of one of the tissues section taken before MALDI
289 chemical imaging with both the adenohypophysis (AH) and neurohypophysis (NH)
290 regions of the pituitary clearly visible. Distribution of AVP (green color) and ACTH (red
291 color) obtained by MALDI chemical imaging (analysis time of 3.5 hrs, 100 μm x 100 μm
292 pixel size) among the different tissue subtypes is shown in Figure 2c. The signal for
293 AVP dominated the NH region while the signal for ATCH was dominant in the AH
294 region. Figure 2b shows a scanned optical image of a sister section of the sample
295 presented in Figure 2a. The 90 surface locations marked with white plus signs are on
296 the nodes of a 9 x 10 grid with 2 mm spacing and represent the locations chosen for
297 analysis by droplet-based liquid microjunction surface sampling-HPLC-ESI-MS/MS.
298 The areas sampled included both the anterior AH and the posterior NH regions of the
299 pituitary as well as regions outside the tissue onto the glass-mounting slide. Given the
300 solvent and extraction conditions, the diameter of a liquid junction at the tissue surface
301 (i.e., the area sampled) at each of these points was about 1.5 - 2 mm. The sample
302 throughput of the system was about 11 min/sample with the time for the
303 chromatographic separation being predominate. For the 90 sampling locations the total

304 analysis time (including samples analyzed outside the tissue margins) was about 16.5
305 hrs. Chromatograms respectively associated with the circled green and red sampling
306 positions in Figure 2b are presented in Figure 2e and 2f for the spatially resolved
307 detection and quantitation of AVP and ACTH. These chromatograms were generated
308 using the SRM transitions for AVP (cpd **1**, *m/z* 543.2 → 328.3, Figure 2e) and ACTH
309 (cpd **2**, *m/z* 757.8 → 876.2, Figure 2f). To automatically calculate compound specific
310 distributions of cpds **1** and **2** (i.e., heat maps), the dropletProbe Premium[®] software
311 was provided with the retention time ranges for background and peak area calculation
312 [29,31,32]. Gray and white sections show the time ranges used for peak integration
313 (R_f =5.6-6.4 min for cpd **1**, R_f =6-6.8 min for cpd **2**) and background calculation (R_f =2-2.8
314 min for both cpds), respectively. It is important to note, that similar to earlier
315 applications,[29,31,32] background-corrected signal only provides a relative distribution
316 of the analyte of interest and does not necessarily reflect the absolute amounts present.
317 The heat maps from the droplet sampling data were overlaid on the optical image to
318 show the relative intensity of the signals detected for AVP and ATCH using green and
319 red bars, respectively (Figure 2d). Comparison of these protein distribution results with
320 those obtained by MALDI chemical imaging (Figure 2c) finds good agreement between
321 the two techniques. In each data set AVP was dominant in the NH region and ACTH
322 dominant in the AH region of the pituitary.

323 *Profiling Multiple Normal and Tumor Human Pituitary Gland Tissues.* The
324 analytical utility of the current droplet-based liquid extraction surface sampling-HPLC-
325 ESI-MS/MS system for potential clinical application was evaluated for the analysis of
326 cadaveric non-tumor pituitary gland thin tissue sections (Pit6, see above), and surgically

327 removed ACTH secreting (coded A3, A4, and A5, obtained from different specimens)
328 and non-secreting (coded N1) pituitary adenomas. A subset of pituitary adenoma,
329 microadenomas, are too small to be detected with diagnostic imaging such as magnetic
330 resonance imaging (MRI), but the presentation of clinical symptoms triggered by the
331 overproduction of pituitary hormones suggests the presence of a pituitary tumor. [38,39]
332 Cushing syndrome is observed in patients affected by ACTH secreting adenomas.[39]
333 The ability to directly localize the tumor during pituitary surgery through the detection of
334 hormone levels differing between malignant and normal tissues would significantly
335 enhance surgical precision and allow preservation of healthy functioning gland.

336 Optical images of H&E-stained samples are shown in Figures 3a-e. Portions of
337 the same tissues are shown with higher magnification images (corresponding to areas
338 marked by white rectangles in Figures 3a-e) in Figures 3f-j (areas of about 0.28 mm x
339 0.28 mm in size) to resolve detailed histological information. Figure 3k shows averaged
340 integrated chromatographic peak areas with standard deviation for AVP and ACTH
341 levels for Pit6 AH and NH regions, normal NH and ACTH secreting malignant regions of
342 sample A3, ACTH secreting A4 and A5 tumor samples and non-secreting N1 pituitary
343 adenomas, and for clean glass slide surface used as blank. (These values are also
344 summarized in Supplemental Table 1.) The green and red dashed lines in the plot
345 indicate background signal levels plus standard deviation for AVP and ACTH,
346 respectively. Three tissue samples at a minimum of two different locations apiece were
347 analyzed for each tissue subtype. Individual sampled locations and corresponding
348 integrated signal levels of AVP and ACTH for samples Pit6, A3, A4, A5 and N1 are
349 shown in Supplemental Figures S2-S6, respectively. Due to their expected chemical

350 variability, results for different regions of samples coded Pit6 and A3 are shown
351 separately in Figure 3k. In agreement with the known histological presentation of
352 normal pituitary gland sample Pit6, ACTH and AVP were found dominant respectively in
353 the AH and NH regions of the gland. Protein levels in the normal NH region of sample
354 A3 were found to be similar to that of the NH region of Pit 6, as expected. However,
355 higher ACTH levels were found in the tumor region of A3 as delineated by
356 histopathology evaluation. In addition, elevated AVP level for this sample indicated the
357 possible NH origin of this malignant tissue. ACTH secreting sample A4, that indicated a
358 highly tumorous structure on the H&E-stained images in Figures 3c and 3h, exhibited a
359 high level of ACTH while AVP signal was negligible. Histopathological evaluation of
360 ACTH secreting sample A5 suggested a similar structure to that of the AH region of
361 normal Pit6 with some indication of tumor penetration in the tissue (see Figures 3f and
362 3i). In agreement with histopathology, protein levels were found to be similar to that of
363 AH region of Pit 6 with ACTH level being about 10x above background level. Finally,
364 highly malignant (see Figure 3j) non-ACTH secreting tumor sample N1 exhibited protein
365 signals that were statistically at background level.

366

367 **CONCLUSION**

368 We here described the profiling of two proteins – AVP and ACTH – from normal
369 human pituitary gland and pituitary adenoma tissue sections using a fully automated
370 droplet-based liquid extraction-HPLC-ESI-MS/MS system. Heat maps of the two
371 proteins of interest recorded with 2-mm-spatial resolution over a cadaveric normal
372 human pituitary gland revealed that AVP was found to be most concentrated in the

373 posterior neurohypophysis and ACTH was found predominantly in the anterior
374 adenohypophysis regions. These results were in good agreement with results obtained
375 by MALDI chemical imaging, and aligned with the known distribution of these hormones
376 in the pituitary. In addition, ACTH and AVP levels obtained from surgically removed
377 ACTH secreting and non-secreting pituitary adenomas agreed with histopathology
378 evaluation of these samples.

379 On the basis of these results and the relative simplicity, rapidity and specificity of
380 the current methodology, the potential exists for this basic technology, with further
381 advancement, to be used to assist surgical decision-making. In the present work,
382 profiling of ACTH and AVP took 11 min per sample with the time for the
383 chromatographic separation being predominate. This timeframe is comparable to that
384 of traditional histopathology evaluation, while providing detailed molecular information
385 currently unattainable during surgery. Switching from HPLC to current state-of-the-art
386 ultra performance liquid chromatography (UPLC) would already offer a 5-8 time
387 decrease in chromatography separation time (current 10 minutes) with concomitant
388 improvement in detection sensitivity and chromatographic resolution [40]. This
389 envisions that separation of all common pituitary proteins with an upper molecular
390 weight of about 24 kDa [41] can be accomplished in about 3-4 min based on our
391 experience separating proteins with a molecular weight around 15-16 kDa [30].

392 More broadly, monitoring proteins levels in a rapid fashion could have numerous
393 additional applications that could be highly transformative for surgery. For instance, the
394 ability to detect specific pituitary hormones may have an application in the evaluation of
395 pituitary tumor resection specimens – particularly those of microadenomas such as

396 those that secrete ACTH and other hormones like prolactin or growth hormone. Such
397 tumors are only millimeters in size and can be difficult for surgeons to visualize and for
398 pathologists to detect by microscopic review of H&E stained frozen sections. Our
399 previous work demonstrated successful sampling of crude cut bulk tissues [32] with
400 sampling locations that differed in height by as much as 1.9 mm, and some samples as
401 narrow as 1 mm. Those results pave the way for testing this automated sampling
402 system in various diagnostic applications including for cancer detection from tissue
403 biopsy samples directly in the operating environment without the need of a skilled
404 operator.

405

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416

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