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Urinary electrophoretic profiles from chronic fatigue syndrome and chronic fatigue syndrome/fibromyalgia patients: a pilot study for achieving their normalization

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Abstract

Aim of our study was to determine if there were distinct, disease-related patterns of urinary analytes in chronic fatigue syndrome (CFS) and chronic fatigue syndrome/fibromyalgia (CFS/FM) compared to normal controls (NC). Urine was collected from these subjects for two consecutive 24 h periods and aliquots were submitted to micellar electrokinetic chromatography (MEKC). To compensate for the differences in peak migration times, these were normalized from the 35 min duration of run to a 100-point scale, and each peak was assigned its normalized time measure. Peak heights were also normalized by dividing the mAU by that of the internal standard (creatinine) and multiplying by 100. MEKC with normalization for peak height and migration time generated comparable results within each of the patient groups. CFS/FM and CFS had significant differences in peaks compared to NC that may be of significance as biomarkers of illnesses.
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1. Introduction

Capillary electrophoresis (CE) is renowned for reproducibly separating mixtures of analytes present in complex biological fluids, such as urine [1–4]. Urine is an ideal fluid for disease screening, since it is readily accessible, can be transported in an intact state by using preservative and freezing and contains metabolites belonging to a wide variety of chemical classes, including purines and/or pyrimidines, organic molecules, carbohydrates and peptides [5]. Although, the application of different electrophoretic techniques [6] has shown that a large number of peaks can be observed and separated in urine specimens, identification of each peak has proven elusive. This is unfortunate, since specific patterns or unique individual analytes could serve as biomarkers of disease pathogenesis or activity [7–10]. Obstacles to this goal include the variations in total migration time, a function of electroosmotic flow (EOF) inside the capillary, of sample loading, wall interactions, physical errors (such as injection irreproducibility and temperature variations) in the methods [11]. The combination of these factors represents a limitation to the precision of electrophoretic procedure and often prevents identification of homologous peaks between prolonged electrophoretic runs. These obstacles may be partially overcome through the "normalization" of electropherograms and a series of efforts have been performed to calibrate electrophoretic profiles and to increase precision of analysis [11–15].

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Urinary electropherograms have never been systematically examined in subjects with chronic fatigue syndrome (CFS) alone and CFS plus fibromyalgia (CFS/FM). CFS is an enigmatic disease characterized by significant fatigue, lasting at least 6 months, associated with at least four of eight ancillary criteria: sore throat, sore lymph nodes, myalgia, arthralgia, headache, neurocognitive and memory dysfunction, sleep irregularities, and extreme fatigue with minimal exertion [16]. FM is a syndrome defined by widespread pain affecting all four quadrants of the body and associated with tenderness to 4 kg of pressure applied to 18 standardized tender points (American College of Rheumatology criteria) [17]. Subjective complaints of fatigue and pain are central to these overlapping diagnoses. Objective criteria, such as substance P concentrations in cerebrospinal fluid and blood analytes have promise, but are not specific or selective for these syndromes [18–21]. This lack of evidence limits insights into pathogenic mechanisms.

Capillary electrophoresis was applied for these pilot studies because of its excellent resolving power. We have used creatinine (Cr) and uric acid (UA) to define recognizable patterns of urine peaks: the data from a series of CE runs were normalized for their creatinine content (normalized peak height, nPh) and migration times (normalized migration time, nMt) so that both qualitative and quantitative comparisons could be made for the entire profile of urinary analytes detected at 214 nm. This issue was examined by comparing electrophoretic patterns of urine samples from normal controls (NC), CFS and CFS/FM patients.

2. Experimental

2.1. Chemicals

Doubly distilled water used for all CE experiments was prepared with a Millipore (Bedford, MA, USA) Milli-Q purification system. All other reagents were of analytical grade and were used without further purification. The urinary creatinine concentration was measured by using both the colorimetric assay kit (Quidel Corporation, San Diego, CA, USA) and the CE determination. In the latter case, a calibration curve was produced, by injecting known scalar amounts of the authentic compound.

2.2. Study population and subject recruitment

This CE investigation examined urine from a subset of subjects who took part in a larger clinical trial. The purpose of the trial was to examine subjective, psychometric, and objective measures in control and chronic fatigue and pain syndromes in order to define clinically relevant subgroups within this diverse population [22]. All participants to this study (56 subjects in total, divided into three groups, aged from 38 to 54 years, mean 46 years) gave their informed consent to volunteer for this paid, Institution Review Board (IRB)-approved protocol. Subjects had a comprehensive screening evaluation to confirm preexisting diagnoses and evaluate co-morbidities [23]. Exclusion criteria included: severe physical impairment, medical conditions with symptoms similar to the subject group under investigation (e.g., morbid obesity, autoimmune/inflammatory diseases, cardiopulmonary disorders), uncontrolled endocrine or allergic disorders, malignancy, severe psychiatric illnesses (e.g., schizophrenia, substance abuse), factors known to affect the hypothalamic–pituitary–adrenal (HPA) axis or autonomic function (cigarette smoking, daily intake of caffeine exceeding the equivalent of two cups of coffee), or medication usage other than as-needed analgesics (excluding long-term narcotics). They were allowed to continue if on stable dosages of thyroid hormone. Where necessary, an appropriate wash-out period of all medications was applied. Medications could only be discontinued for 3 days. This was mandated by our IRB, since they felt a longer period was an unethical discontinuation of essential medications. Acetaminophen and diphenhydramine were permitted on an as-needed basis for pain and sleep, respectively, until 3 days prior to the study visit. Subjects had a history and physical examination, were administered the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (SCID II) [24] and Composite International Diagnostic Interview (CIDI) [25,26] to detect psychiatric co-morbidities. They completed the self-report Short Form 37 (SF-37) [27,28], Self Efficacy Scale (SES) [29], Meaning of Illness Questionnaire (MIQ) [30], and Multidimensional Fatigue Inventory (MFI) [31].

The population investigated consisted of normal healthy controls ($n = 20$; 10 males and 10 females), chronic fatigue syndrome (CFS) patients ($n = 11$; 5 males and 6 females) [16], and subjects with chronic fatigue syndrome/fibromyalgia (CFS/FM) ($n = 25$; 12 males and 13 females). The two groups of patients included two Persian Gulf War veterans with CFS, and 8 with CFS/FM [32]. Subjects were admitted to the Georgetown University General Clinical Research Center (G-CRC) on the evening of Day 1. An 18-gauge catheter was inserted antecubitally and infused with normal saline at 50 ml/h to ensure adequate hydration of all subjects. Urine collections (24 h) ended on admission (Day 1) and began again the morning of Day 2 when the series of physiological stressors were given [23]. These stressors included quantitative thumbnail pressure-and forearm heat-induced pain (Stressor I) [33]; cognitive challenges of visual, spatial, short term and verbal working memory (Stressor II); isometric hand grip to test autonomic function and muscular fatigue (Stressor III) [34]; sub-maximal exercise test on an electronically-braked cycle ergometer (Sensormedics, Yorba Linda, CA) (Stressor IV); and lumbar puncture (Stressor V). Boric acid (15 g for 24 h urine collection) was used as a preservative. Urine volumes, urine and serum creatinine concentrations were measured. Urine aliquots were frozen at −80 °C without processing until thawed for CE analysis [35,36]. Prior to injection,

samples were shaken for 1 min and filtered through a 45 μm filter (Whatman, Clifton, NJ, USA).

2.3. Capillary electrophoresis equipment

Electrophoretic analyses were performed with a P/ACE 2100 instrument (Beckman, Palo Alto, CA, USA) interfaced to a Beckman P/ACE, version 1.21 data station, and equipped with a UV detector.

All coated (hydrophobic coatings C1, and C18; hydrophilic coatings P175 and P150) capillaries (total length 57 cm; effective length 50 cm; 50 μm i.d.) were obtained from Supelco (Bellefonte, PA, USA). Uncoated fused-silica capillaries (total and effective length 57 and 50 cm, respectively; 50 μm i.d.; 375 μm o.d.) used for MEKC runs were from Polymicro Technologies (Phoenix, AZ, USA) and were operated at 20 ± 0.1 °C by means of a cooling liquid circulating continuously through the cartridge.

Samples (ca. 10 nl) were introduced in pressure injection mode for 10 s at 0.5 psi (1 psi = 6894.76 Pa). Analyses were run at 20 kV using a cartridge detection window of 100 μm × 800 μm. CZE experiments were performed using 50 mM sodium tetraborate pH 9.0 as background electrolyte. For MEKC experiments, runs were carried out essentially as previously described [37] in 50 mM sodium tetraborate, pH 9.3 containing 65 mM SDS. Analytes were monitored at 214 nm. At the beginning of each working day, the capillary was washed with water, 0.1 M NaOH, water and separation buffer for 4 min. The capillary was rinsed between runs sequentially for 3 min with water, 0.1 M NaOH, and then with water and buffer solution. Migration was positive to negative polarity.

2.4. Normalization of CE profiles

As is generally the case, the heights and migration times for each peak varied between samples, although the patterns were reproducible for individual samples.

2.4.1. Normalization of migration times

The time required to move all the material in a given sample depends on many factors but the order of analyte migration does not change as long as the same method is used consistently.

The electrophoretic profiles obtained from specimens analyzed in this study contained, in addition to two major peaks (that were confirmed to be creatinine and uric acid by co-injecting exogenous standards), distinct tall peaks, reproducible for all electropherograms, and a series of multiple, smaller peaks with signal to noise ratios >3. Once that electropherograms showing the optimal migration of the above peaks were inspected and migration time measured for each peak, these times were normalized by dividing migration time (min) by the full 35 min duration of each run and multiplying by 100. The result was a template with each peak assigned its normalized migration time. The coded patterns of peaks from the other profiles were then inspected and nMt assigned to the appropriate peaks using the template. Each electropherogram was inspected and the tall creatinine and uric acid peaks identified. A second strata of moderately sized, reproducibly eluted peaks were then located (peaks 10, 20, 30 and 40; see below). The smaller, intervening peaks with signal to noise ratios >3 were identified and labeled. These coded peaks were then assigned their nMt based on the idealized template.

2.4.2. Normalization of peak heights

The baseline was arbitrarily assigned a "peak height" of 0.25 mm (width of ink tracing) and converted to $mAU_{214\,nm}$ to permit logarithmic transformations. The heights of each peak ($mAU_{214\,nm}$), including creatinine, varied widely between individuals and could not be directly compared. Peak heights of each electropherogram were normalized by dividing each peak height by that of creatinine and multiplying by 100. As a result, all creatinine spikes had a normalized peak height of 100. Thus, using these two systems, we converted the raw MEKC migration data from the independent time series of peaks of each sample into data sets that were normalized for both peak heights and migration times. The reproducible peaks were given numbers 1–47 as described in the results section.

2.5. Statistical analysis

Samples were chosen so that age and gender distributions were as comparable as possible. Urine samples were analyzed in two batches approximately 6 months apart. Identical methods were used for both batches. The first pilot batch contained Days 1 and 2 samples from CFS/FM, CFS and NC subjects. Samples for the second batch were essentially the same as those of batch one minus a few ($n = 5$) urine specimens that unfortunately thawed in transit and were discarded.

The normalized electrophoretic data for each peak were compared within each of the three groups. Differences between Days 1 and 2 were compared by two-tailed paired Student's *t*-tests with equal variance for each peak (nPh). There were no significant differences in any group, indicating reproducible electropherograms from Days 1 and 2 samples. Therefore, all the Days 1 and 2 MEKC results were combined to give 25 CFS/FM, 11 CFS and 20 NC datasets. The geometric mean nPh (and 95% confidence intervals) was determined for each peak within the three groups. nPh data were compared between groups by an analysis of variance (ANOVA). This included data from runs where peaks were absent and the nPh for the baseline was assigned (described above). If significant ($p < 0.05$), comparisons between pairs of groups were performed by two-tailed unpaired Student's *t*-tests. Significance was ascribed for $p < 0.05$.

The prevalences of each peak on Days 1 and 2 within the three groups were compared by χ^2 testing. This was to

determine if peaks were absent or only infrequently present in any group, the Day 2 stressors, and if their absence could be related to the disease states. The theoretical possibility that urine peptide profiles were related to prior drug use is acknowledged, but does not detract from the concept used for analysis of the data. When we can identify the specific peaks, one of top priorities will be to determine if they are true positive/disease related or potentially false positive/treatment related.

3. Results

Although, capillary zone electrophoresis (CZE) was characterized by its excellent resolving power, peaks could not be easily identified by their patterns of migration. The hydrophilic capillaries gave excellent peak separation, but the separations were not always reproducible, which reduced their reliability (data not shown). An additional problem was the concern that these columns retained highly adherent analytes from previous runs. Runs performed using capillaries with hydrophobic coating were unsuccessful since these retained most analytes and eluted only 1 or 2 peaks in 35 min (data not shown). Separation by MEKC was most successful and reproducible. The electrophoretic profiles representative of each group of subjects investigated (i.e., trace a for NC; traces b and c for CFS and for CFS/FM groups, respectively), obtained from samples collected on day 1 are shown in Fig. 1. Samples run on 3 consecutive days, refrozen, and rerun again 6 months later gave virtually identical electropherograms (not shown). However, since the mobility and spread of the peaks differed from specimen to specimen, to avoid that the mere observation of electrophoretic profiles could generate distortions in the identification of analytes in the various groups of subjects investigated, these patterns were normalized for both peak height and migration time, as described in the experimental section. Once normalized, consistent qualitative patterns became readily apparent. Creatinine was the first and highest peak on each run: its normalized peak height was placed is equal to 100, its migration time was ∼6 min, and the normalized migration time is equal to 17.8. Other peaks observed in the electropherograms have been indicated with the numbering 1–47 and listed in Table 1. In addition to these 48 peaks, there was a number of low-magnitude peaks in CFS/FM and CFS profiles that did not appear in the NC pattern. These were not further assessed because of the low signal-to-noise ratios. The peak identification nomenclature indicated above was only applicable to the conditions used in this experiment.

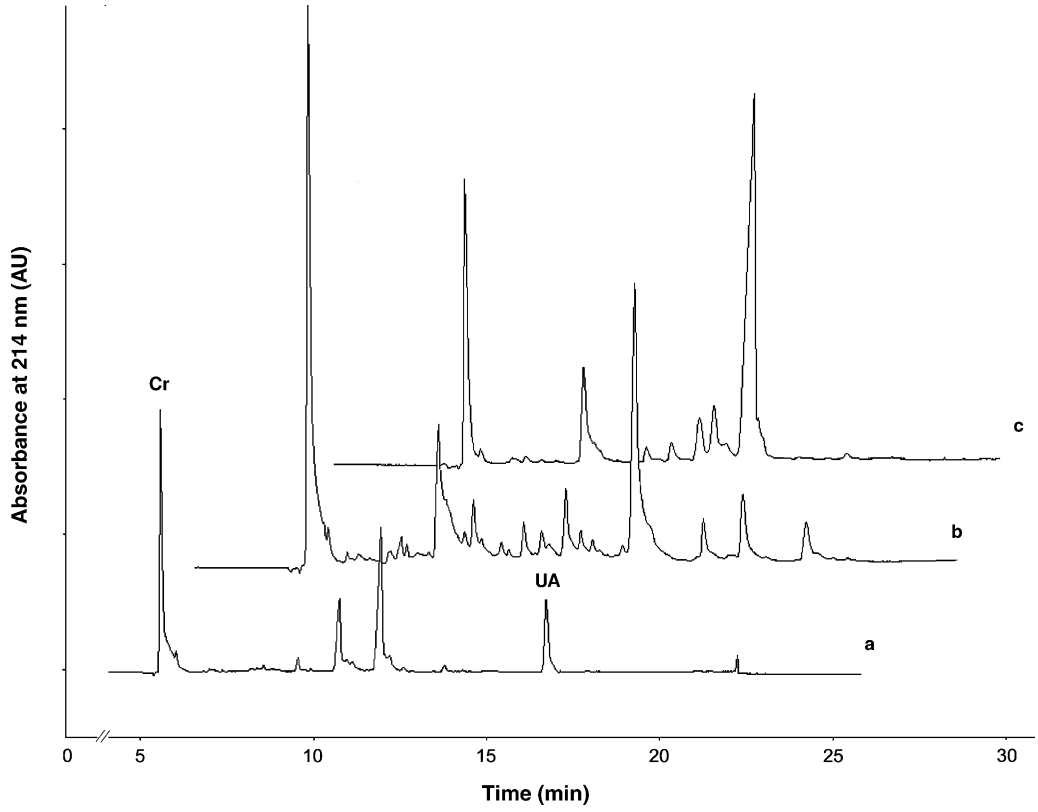

Fig. 1. Micellar electrokinetic chromatographic profiles representative of the urinary pattern in normal controls (NC) (trace a), in chronic fatigue syndrome (CFS) and chronic fatigue syndrome/fibromyalgia (CFS/FM) patients (traces b and c, respectively). *Experimental conditions*: runs were performed in uncoated fused-silica capilarries (50 cm effective length; 50 μm i.d.) using 50 mM sodium tetraborate, pH 9.3 containing 65 mM SDS as a running buffer, at a constant voltage of 20 kV. Samples (ca. 10 nl) were introduced in pressure injection mode for 10 s at 0.5 psi. The peaks indicated by Cr and UA in all traces are creatinine and uric acid, respectively.

Table 1
Left-peak numbering (1–47) and normalized migration times (nMt) of peaks observed in electrophoretic profiles of normal controls (NC), chronic fatigue syndrome/fibromyalgia (CFS/FM) and chronic fatigue syndrome (CFS) patients

Electrophoretic peaks		Prevalences of peaks		
Peak number	Normalized migration time (nMt)	NC	CFS/FM	CFS
1	21	20/20 (100%)	**17/25 (68%)*****	11/11 (100%) §§§
2	22.1	17/20 (85%)	20/25 (80%)	9/11 (82%)
3	23	13/17 (65%)	15/25 (60%)	4/11 (36%) §
4	24	**15/20 (75%)** †	22/25 (88%)	10/11 (91%)
5	24.7	20/20 (100%)	25/25 (100%)	11/11 (100%)
6	25.5	8/20 (40%)	13/25 (52%)	**2/11 (18%)** §§
7	*27.8*	*5/20 (25%)*	*6/25 (24%)*	*2/11 (18%)*
8	29.1	18/20 (90%)	23/25 (92%)	11/11 (100%)
9	29.8	2/20 (10%)	**14/25 (56%)*****	2/11 (18%) §§§
10	*30.4*	*4/20 (20%)*	*4/25 (16%)*	*2/11 (18%)*
11	*30.9*	*3/20 (15%)*	*2/25 (8%)*	*0/11 (0%)*
12	31.1	**16/20 (80%)** †,*	25/25 (100%)	11/11 (100%)
13	31.6	10/20 (50%)	13/25 (52%)	**3/11 (27%)** †,§
14	*32*	*3/20 (15%)*	*9/25 (36%)*	*2/11 (18%)*
15	*32.5*	*0/20 (0%)*	*2/25 (8%)*	*2/11 (18%)*
16	*32.8*	*0/20 (0%)*	*1/25 (4%)*	*0/11 (0%)*
17	*33*	*0/20 (0%)*	*2/25 (8%)*	*0/11 (0%)*
18	33.3	20/20 (100%)	25/25 (100%)	11/11 (100%)
19	*34*	*4/20 (20%)*	*5/25 (20%)*	*1/11 (9%)*
20	35.6	19/20 (95%)	25/25 (100%)	9/11 (82%)
21	36	7/20 (35%)	5/23 (22%)	**0/11 (0%)** §
22	*36.2*	*2/20 (10%)*	*1/23 (4%)*	*0.11 (0%)*
23	*36.5*	*2/20 (10%)*	*1/23 (4%)*	*0/11 (0%)*
24	36.8	11/20 (55%)	12/23 (52%)	**2/11 (18%)** †††,§§
25	38.0	20/20 (100%)	23/23 (100%)	11/11 (100%)
26	38.7	18/19 (95%)	17/23 (74%)	**8/11 (73%)**
27	41.8	14/16 (88%)	22/23 (96%)	**8/11 (73%)** §§§
28	42	11/15 (73%)	16/22 (73%)	9/11 (82%)
29	*45.5*	*0/14 (0%)*	*1/21 (5%)*	*1/11 (9%)*
30	47.1	9/14 (64%)	15/21 (71%)	8/11 (73%)
31	*47.7*	*0/12 (0%)*	*2/21 (10%)*	*1/11 (9%)*
32	*47.9*	*0/12 (0%)*	*1/21 (5%)*	*0/11 (0%)*
33	49.3	9/12 (75%)	16/21 (76%)	**10/11 (91%)** †
34	50	0/12 (0%)	3/21 (14%)	**3/11 (27%)** †
35	50.6	0/12 (0%)	1/21 (5%)	**2/11 (18%)** §
36	*51*	*0/12 (0%)*	*2/21 (10%)*	*0/11 (0%)*
37	51.6	12/12 (100%)	20/21 (95%)	11/11 (100%)
38	52	0/11 (0%)	1/21 (5%)	**4/11 (36%)** ††,§§§
39	55.8	**8/11 (73%)** †,*	19/21 (90%)	10/11 (91%)
40	71.15	11/11 (100%)	20/20 (100%)	11/11 (100%)
41	75.5	**10/10 (100%)****	12/18 (67%)	5/10 (50%)††
42	80.9	8/9 (89%)	16/17 (94%)	7/10 (70%)
43	82.1	**4/9 (44%)** †††	5/17 (29%)	1/10 (10%)
44	*82.5*	*1/9 (11%)*	*2/17 (12%)*	*0/10 (0%)*
45	87.1	9/9 (100%)	17/17 (100%)	10/10 (100%)
46	*87.4*	*0/4 (0%)*	*2/11 (18%)*	*0/7 (0%)*
47	96.9	4/4 (100%)	11/11 (100%)	7/7 (100%)

Bold numbers indicate peaks with a uniformly high prevalence; numbers in italics indicate peaks with a uniformly low prevalence; all other numbers indicate peaks with significance differences between groups. Right-Prevalence of each peak for each group of electropherograms and significant differences by χ^2 testing. Numbers in bold indicate the prevalences that were the most significantly difference between groups. Probability by χ^2: NC vs. CFS/FM, (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$; NC vs. CFS, (†) $p < 0.05$, (††) $p < 0.01$, (†††) $p < 0.001$; CFS/FM vs. CFS, (§) $p < 0.05$, (§§) $p < 0.01$, (§§§) $p < 0$.

There were no differences in the peak areas (as determined from the $mAU_{214\,nm}$ absorbance) for creatinine between the paired Days 1 and 2 samples for each subject and in each of the groups. This was consistent with the urine creatinine concentrations. As shown in Fig. 2, urine creatinine concentrations (determined with the colorimetric assay mentioned in the experimental section) and $mAU_{214\,nm}$ measurements were closely related. The explained variance (R^2) was 0.63 for linear regression analysis of all specimens. The urine creatinine concentration was significantly higher for CFS (1.62 mg/ml; 1.06–2.18, 95%CI) and NC (1.23 mg/ml; 1.02–1.44) than CFS/FM (0.78 mg/ml; 0.64–0.92, $p < 0.001$

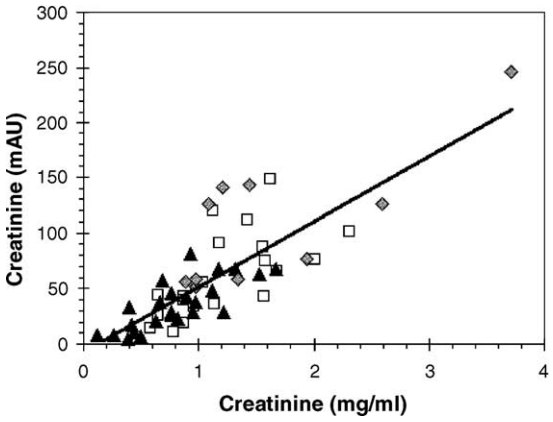

Fig. 2. Relationship between urinary creatinine concentrations determined using the capillary electrophoretic measurement at 214 nm (*y*-axis) and the colorimetric assay at 490 nm (*x*-axis). Linear regression data are shown for NC (open squares), CFS/FM (solid triangles) and CFS (grey diamonds) ($R^2 = 0.63$ for all points).

compared to each by two-tailed unpaired Student's *t*-test). The same was true for $mAU_{214\,nm}$ for CFS (93.6; 56.6–130.5) and NC (61.6; 44.8–78.4) compared to CFS/FM (35.8; 26.8–44.7), $p < 0.01$). There were no significant differences between means or linear regression results within or between the CFS/FM, CFS or NC groups, males versus females, or gender subsets of each group. The majority of males from all groups had creatinine levels clustered at ≤ 1.5 mg/ml. The CFS group had one male and two female outliers (>the 95%CI from the overall regression line), while the NC group had one male and one female outlier. Their creatinine concentrations were between 1.1 and 1.7 mg/ml.

Negative interference of the urine creatinine measurements was the most likely explanation [38]. Bilirubin, saccharides, catecholamines, denatured hemoglobin (if perimenstrual), carbonyl compounds, and metabolites of pyridium (used to treat bladder irritation in CFS) and herbal remedies were potential interfering substances. These are unlikely to interfere with $mAU_{214\,nm}$ detection of creatinine after CE. Removal of these probable false negative results increased the explained variance (R^2) to 0.80. An alternative explanation was that the pathophysiological process of CFS evoked excretion of a substance that interfered with the urine assay method.

3.1. Differences between groups

Each normalized peak height was compared between Days 1 and 2 urine samples for each of the groups. The datasets were averaged (geometric means) to give the normalized migration profiles reported in Fig. 3 for NC (trace B), CFS and CFS/FM groups (traces A and C, respectively).

Quantitative comparisons (Fig. 4) identified six CFS/FM peaks that were significantly higher (peaks 4, 5, 9, 12, 18 and 25), and two that were lower (peaks 1 and 21), than NC. CFS/FM was significantly higher than CFS for peaks 9, 24, 25, 27, and 32. CFS was significantly greater than NC at peaks

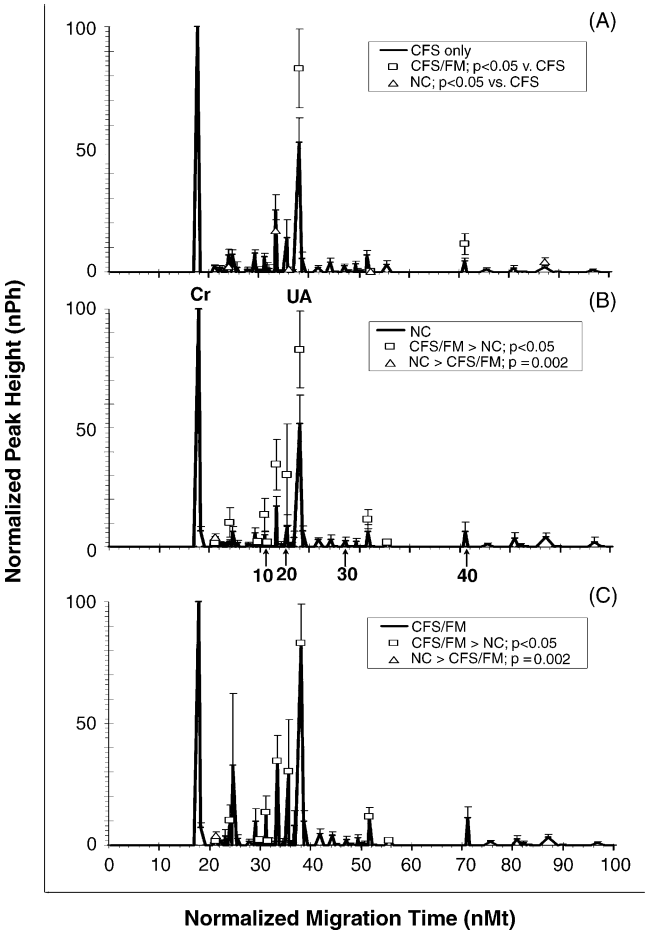

Fig. 3. Overlapping of normalized MEKC patterns for normal controls (NC) (trace B), chronic fatigue syndrome (CFS) and chronic fatigue syndrome/fibromyalgia (CFS/FM) patients (traces A and C, respectively). The tens line in trace B indicates the position of peaks: 10; 20; 30 and 40, respectively. The mean normalized peak heights (nPh) are shown as the short bars at the peaks with upward 95% CI error bars and connected by the dark line. Open squares (with 95% CI) indicate nine peaks where the mean CFS/FM results were significantly greater than NC, and one where the NC result was higher. Open squares (with 95% CI error bars) for CFS/FM and open triangles for NC represent nPh that were significantly different from the CFS results.

4 and 38, and lower for peaks 21 and 24. These rankings were further refined to determine if patterns of peaks were associated with CFS/FM or CFS. The CFS/FM and CFS groups shared elevated peaks 4, 17 and either 37 or 38 compared to NC. Peak 37 was low, but was significantly ranked in terms of nPh as CFS/FM > CFS > NC. These peaks may reflect some common factors associated with fatigue and CFS.

These results identified potential markers of CFS/FM and CFS status. The peaks 9 and 25 were significantly higher in CFS/FM than both CFS and NC. These may represent markers of FM or the combination of CFS and FM. Other potential markers of FM that were higher in CFS/FM than CFS were 24, 27 and 32. CFS/FM also had higher peaks 5, 12 and 18 than NC. Both, CFS/FM and FM had higher peak 4 levels than NC, suggesting that this may be a marker of CFS.

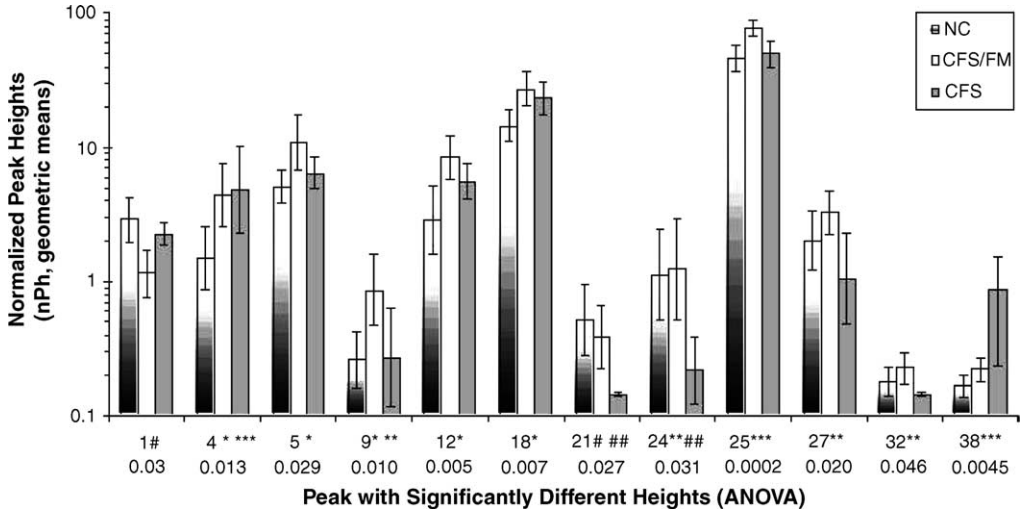

Fig. 4. Peaks with significantly different heights. Normalized peak heights (nPh) were plotted for those peaks showing a significant difference by ANOVA between the geometric means for the three groups. Peak identification codes and ANOVA values were shown under each set of three bars. The first bar (black at the bottom grading to white at the top) represented NC; the middle white bar CFS/FM; and the third grey bar CFS results. The error bars were 95% CI. Peak N2 data were arithmetic means. Significant differences between groups ($p < 0.05$, two-tailed unpaired Student's *t*-tests) were annotated as (*) CFS/FM > NC; (**) CFS/FM > CFS; (***) CFS > NC; (#) N > CFS/FM; and (##) N > CFS.

Peak 38 of the CFS group was also significantly elevated above NC. Another potential marker was the virtual absence of peak 21 in the CFS/FM and CFS groups compared to NC. Peak 1 was higher in NC than CFS/FM. Peak 24 was higher in NC than CFS.

3.2. Prevalences of peaks

A variable that could affect these averaged peak heights was the prevalence of each peak in Days 1 and 2 runs for each group. This was because the geometric means incorporated baseline values when no peaks were detectable. A decrease in the prevalence of peaks would lead to more baseline values and a lower average. The NC group had no significant differences in the prevalences of each peak between Days 1 and 2. Both the CFS/FM and CFS groups had fewer positive results for Peak 26 on Day 2 than Day 1 (from 11/12 to 6/11 and 5/5 to 3/6, respectively; $p < 0.05$ by χ^2). The CFS group had an increase in peak 13 from 0/5 to 3/6 ($p < 0.05$), while the CFS/FM group has an increase from 3/12 to 6/13 (not significant). When the CFS/FM and CFS group data were combined, the increase in 13 ($p < 0.05$) and decrease in 26 ($p < 0.01$) were significant between Days 1 and 2. Peaks 13 and 26 may be markers of the Day 2 stressor responses of these CFS/FM and CFS subjects.

As shown in Table 1, qualitative assessment of the combined Days 1 and 2 data revealed that peaks 2, 5, 8, 18, 20, 25, 26, 28, 30, 37, 40, 42, 45 and 47 had high prevalence in all three groups. These may be considered normal urine constituents not altered by CFS or FM mechanisms. Peaks that had higher prevalence in CFS/FM were 9 (five-fold higher), 12 and 39. Peaks that were more prevalent in CFS than NC included 4, 12, 33, 38 and 39. The presence of 12, 39 and the less prevalent 34 peak may be indicators of CFS. Peak 9 was significantly more prevalent in CFS/FM than NC and CFS, suggesting that it may be a marker of the fibromyalgia component of CFS/FM. Peaks with higher prevalences in NC than CFS/FM were 1, 41 and 43. Prevalences were higher in NC than CFS for 3, 6, 13, 21, 24, 27 and 41. The absence of these peaks could be a significant component of the overall changes in urine analytes for CFS/FM and CFS compared to NC.

The remaining peaks were of low prevalence in all three groups, and may represent unique urine components from specific individuals.

4. Discussion

The order of the major peaks in CFS/FM, CFS, and NC urine samples was maintained, indicating that similar analytes were excreted in all samples analyzed. The impressively reproducible separation of these peaks was an indication of the capabilities of MEKC methodology. Analytes present in patients' groups but not in normal subjects suggest differences in the metabolism or origins of these materials. They may reflect differences in plasma concentrations; filtration (autonomic control of renal glomerular blood flow); renal tubule peptidases, reabsorption or secretion; or even bladder epithelial origins. If CFS represents a systemic illness with proteomic changes in multiple cell types (e.g., platelets and muscle [39–44]) then the tissues immediately responsible for the analytes present in the urine or their chemical modification may offer novel clues into disease pathophysiology.

The creatinine concentrations from the 24 h urine collections were significantly lower in the CFS/FM than NC and CFS groups (Fig. 2). This suggests difference in net free water intake or clearance, or low muscle mass in the CFS/FM

group. Despite this, CFS/FM had more peaks and significantly higher peaks than NC (Fig. 3, trace c). This indicated that CFS/FM had increased numbers and concentrations of analytes relative to NC even though they may have had more dilute urine (significantly lower creatinine). This observation is under further study.

A key difference between our analysis and other assessments of urine analytes in disease states was the normalization of peak heights to the amount of creatinine detected and normalization of the peak migration time. Once the stereotypic pattern of urine analytes became apparent, it was possible to make comparisons in normalized peak heights between subject groups, and to infer potential biomarkers of CFS and FM pathophysiological processes. Without this normalization, differences can be appreciated (e.g., between osteoarthritis and rheumatoid arthritis) [45] but not quantified.

One major limitation of our procedure was the detection system. The single absorbance measurement, in fact, could not positively identify any of the analytes. Creatinine and uric acid were known from previous experiments with spiked urine [46]. Moreover, the dense overlap of peaks leading to the elevated baseline encountered in CFS/FM and CFS may have generated superimposed, overlapping spectra from the numerous individual urine chemicals that may co-elute in a single peak. These peaks may represent peptides, other amides (e.g., uric acid, creatinine) or other chemicals absorbing in the far UV range. As a result, the identities and pathophysiological sources of the analytes from CFS/FM and CFS subjects that were most different from NC remain elusive. Other detection systems, such as photodiode arrays and/or mass spectrometry would have certainly contributed to define better the physico-chemical characteristics for each peak. In particular, in recent years mass spectrometry technology has entered novel screening and application areas, such as clinical diagnostic and predictive medicine. With its capability of detecting in body fluids changes in analyte's levels that may reflect changes in the disease states, this technique has proven very useful for the human health care community.

In this context, given that the problems connected with the presence of SDS micelles can be overcome, we hope that a decisive methodologic progress in the identification of possible urinary markers of CFS/FM will be achieved applying a current experimental procedure, such as capillary electrophoresis combined to matrix assisted laser desorption/ionization-time-of-flight, an approach that could enable investigators to analyze, in parallel, a large number of analytes in biological fluids.

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