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Determination of peptides and proteins in human urine with capillary electrophoresis–mass spectrometry, a suitable tool for the establishment of new diagnostic markers

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Abstract

The on-line coupling of capillary electrophoresis (CE) with electrospray-time-of-flight mass spectrometry (MS) has been used to obtain patterns of peptides and proteins present in the urine of healthy human individuals. This led to the establishment of a ‘‘normal urine polypeptide pattern’’, consisting of 247 polypeptides, each of which was found in more than 50% of healthy individuals. Applying CE–MS to the analysis of urine of patients with kidney disease revealed differences in polypeptide pattern. Twenty-seven polypeptides were exclusively found in samples of patients. Another 13, present in controls, were missing. These data indicate that CE–MS can be applied as powerful tool in clinical diagnostics.
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1. Introduction

Proteomics gains increasing importance as a new tool for identification of therapeutic targets [1,2]. One of the ultimate challenges is to gain insight into the proteins found in body fluids such as urine and blood. This information could subsequently be used to define the state of health of an individual [3]. We set out to combine the high-resolution properties of capillary electrophoresis (CE) with the powerful identification ability of mass spectrometry (MS). CE is known as an efficient and rapid separation technique with high resolution, which is frequently employed in the separation of biological macromolecules such as proteins [4], peptides [5] and nucleic acids [6]. MS is currently one of the most powerful techniques to analyze biological samples, allowing identification based on the accurate mass of the molecules [7–13]. Therefore, the on-line coupling of CE and MS offers an attractive alternative to common chromatographic separation techniques. The advantages of CE–MS are speed, high resolution, sensitivity, and reproducibility [14]. To date, CE is applied to the separation of proteins [15,16], but the combination of CE and MS to analyze polypeptide patterns in human body fluids is not yet established.

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To date, only a few single proteins or peptides were investigated with CE–MS [17–20]. We have developed an on-line combination of CE–MS to depict polypeptides found in body fluids under the assumption that this will lead to the establishment of polypeptide patterns typical for the state of health of individuals.

Urine analysis provides a fast, non-invasive diagnostic tool for patients with renal diseases. Healthy individuals excrete less than 150 mg/day of protein in urine, while the urinary protein excretion of patients with renal diseases may exceed several grams a day [21]. Thus, the evaluation of these proteins may lead to an increased understanding of renal physiology and possibly allows the differentiation of subgroups of renal diseases by the identification of these proteins via CE–MS. Such data would eventually even make renal biopsy superfluous.

Here we show that the simultaneous assessment of a large number of proteins and peptides in urine is possible and leads to different, distinct polypeptide patterns in renal diseases.

2. Experimental

2.1. Sample preparation

Best reproducibility of results without much degradation was found when spontaneous urine samples were analyzed repeatedly. Spontaneous urine samples of patients and healthy volunteers were obtained after informed consent and stored at −20 °C until further analysis. Urine samples were thawed and 2 ml was applied onto a Pharmacia C_2 column (Amersham Biosciences, Buckinghamshire, UK) to remove urea, electrolytes, salts and other interfering components, to decrease matrix effects and to enrich the polypeptides present. Polypeptides were eluted with 50% (v/v) acetonitrile (Sigma–Aldrich, Taufkirchen, Germany) in HPLC-grade water (Roth, Karlsruhe, Germany) containing 0.5% (v/v) formic acid (Sigma–Aldrich). The pretreated samples were lyophilized and resuspended in 20 μl of HPLC-grade water shortly before use.

2.2. CE–MS

The CE system was a Beckman P/ACE MDQ system (Beckman-Coulter, Fullerton, CA, USA) coupled to a Mariner time-of-flight (TOF) mass spectrometer from Applied Biosystems (Applied Biosystems, Farmington, MA, USA). The sample was injected hydrodynamically (6.9 kPa, 10 s, injecting approximately 100 nl of the sample solution) on an untreated silica capillary (Beckman, 90 cm×75 μm I.D.×360 μm O.D.). Complete electrophoretic runs take about 60 min, using a running buffer composed of 30% (v/v) methanol and 0.5% (v/v) formic acid (pH 2.4) in water. The separation conditions were 30 kV with 1.4 kPa positive pressure and a current of 13 μA. After each run, the CE capillary was rinsed for 5 min with 0.1 *M* NaOH, followed by 5 min rinsing with water and another 5 min with running buffer. The capillary temperature was held constantly at 35 °C.

The electrospray ionization (ESI) interface was from Agilent Technologies (Palo Alto, CA, USA). The sheath flow was applied at 5 μl/min coaxial to the capillary and the sheath liquid was identical to the running buffer described above. The Spray Tip Potential was set to +3500 V. The mass spectrometer used was a Mariner ESI-TOF-MS system, which provides a resolving power of up to 7000.

2.3. Data processing

The data of the mass analyzer was acquired by the Mariner Control Panel software (Applied Biosystems), and the obtained raw data files were initially examined utilizing the Mariner Data Explorer software. Subsequently, the raw data was analyzed using the MosaiquesVisu software for further interpretation of the mass spectra. Initially, CE–MS peaks were detected, then the charge of each peak was calculated, based on isotopic distribution and conjugated peaks. Finally the conjugated peaks were summarized in one single peak and the real masses were calculated. Cut off point for analysis was a relative molecular mass (M_r) less than 1000. The data were deposited in an MS-Access database to allow comparison of individual runs as well as individual samples.

3. Results and discussion

3.1. CE–MS conditions

As described in more detail by Kaiser et al. [22], we have developed a stable on-line combination of CE–MS to analyze the polypeptides present in body fluids. As shown in Fig. 1, more than 1000 polypeptides can be detected in the urine of a healthy individual with this approach. The overall pattern and the mass/charge ratios observed by repeated screening of the same sample as well as in comparison to other individuals of the same group were highly reproducible. The reproducibility of the migration time was less satisfactory, most likely due to precipitation of analytes on the capillary wall. Thus, we decided to test different forms of capillary coating as described in the literature [17,19,20,23,24] to solve this problem. Initially, aminopropylsilylane (APS)-coated capillaries were prepared as described by Moseley et al. [23]. This coating in fact resulted in reproducible migration times for about 10 subsequent sample injections. However, the migration times prolonged again with increasing injections, most likely due to the deposition of compounds from the sample on the coating/column wall. Flushing the column with 0.1 *M* NaOH could reverse this, but also completely removed the coating.

As a next step, we used polyvinylalcohol (PVA)-coated capillaries, which are resistant to NaOH, thus only removing the deposit from the capillary. As reported earlier, this coating led to an excellent separation and to reproducible CE runs when protein/peptide standards were separated ([24] and own observation). However, this unfortunately was not the case when a complex sample obtained from

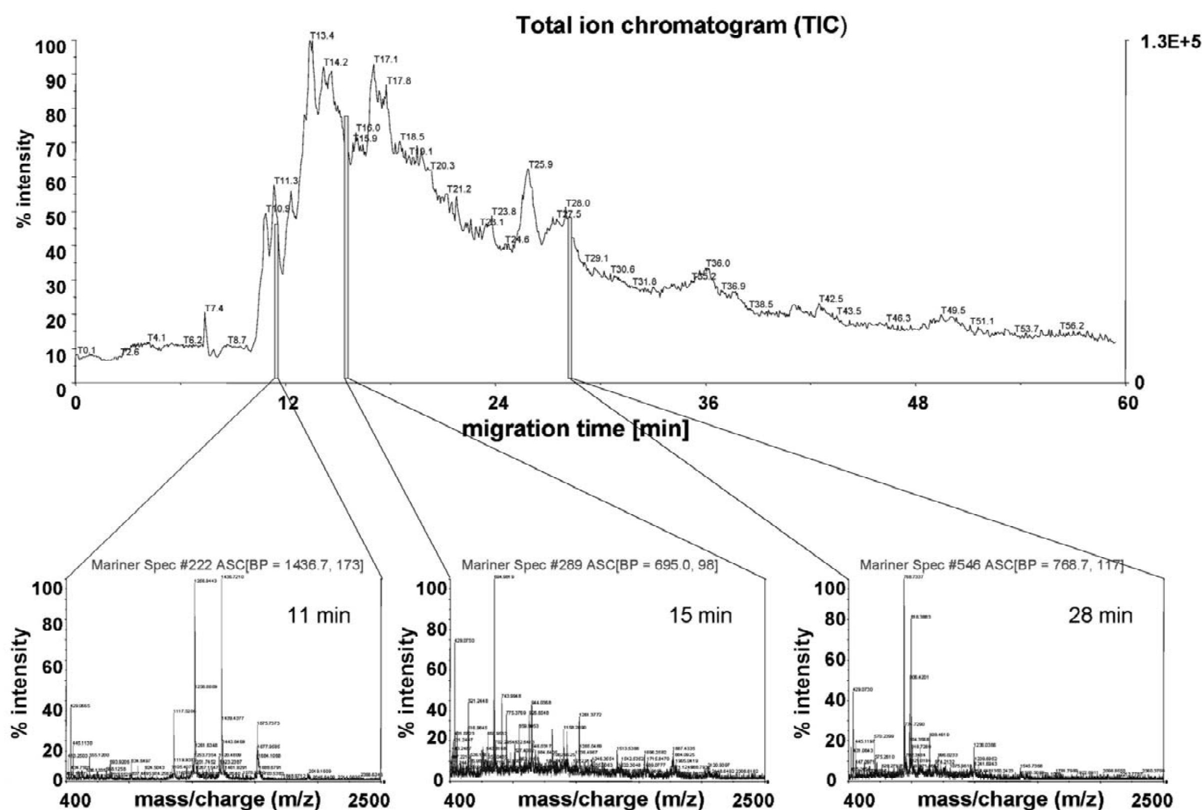


Fig. 1. CE–MS spectrum of urine from a healthy volunteer. The upper panel shows the total ion current (TIC), the lower panel shows three different sections of the CE–MS run at 11, 15 and 28 min.

biological fluids (e.g., urine) was examined. Hence, the use of uncoated capillaries thoroughly cleaned with 0.1 *M* NaOH after each run finally gave the best reproducible results.

As also evident from Fig. 1, the complex data obtained from a single CE–MS analysis cannot be processed using the software, which is supplied with the mass spectrometer. Hence, software solutions to extract the peak information from a CE–MS analysis were developed.

3.2. Data processing and evaluation

3.2.1. Overview

To analyze the vast amount of data produced in a single CE–MS run (Fig. 2) we developed software tailored to the requirements of our analysis. Typically, the raw data consists of 500–1000 time-sequenced mass spectra, 80 000 data points each. These spectra feature gaussian and non-gaussian noise as well as significant baseline offset, produced by unidentifiable analytes across the detection range from 400 to 2500 (m/z) (Fig. 2A).

In a first step, the individual spectra are searched for polypeptide signatures to yield some 100 000 raw peaks. Polypeptides must extend across several successive spectra (we typically require five spectra during data evaluation) and are collected in a second step to yield complete CE–MS peaks, characterized by their position $x=m/z$, $y=$migration time, and amplitude A (Fig. 2B).

In the final step, we attempt to determine unique masses for all polypeptides found and reduce the CE–MS spectrum to isotope-free polypeptides of unit charge, i.e., well defined atomic mass (Fig. 2C). A brief description of the key-points of our polypeptide detection is outlined below.

3.2.2. Matched filtering

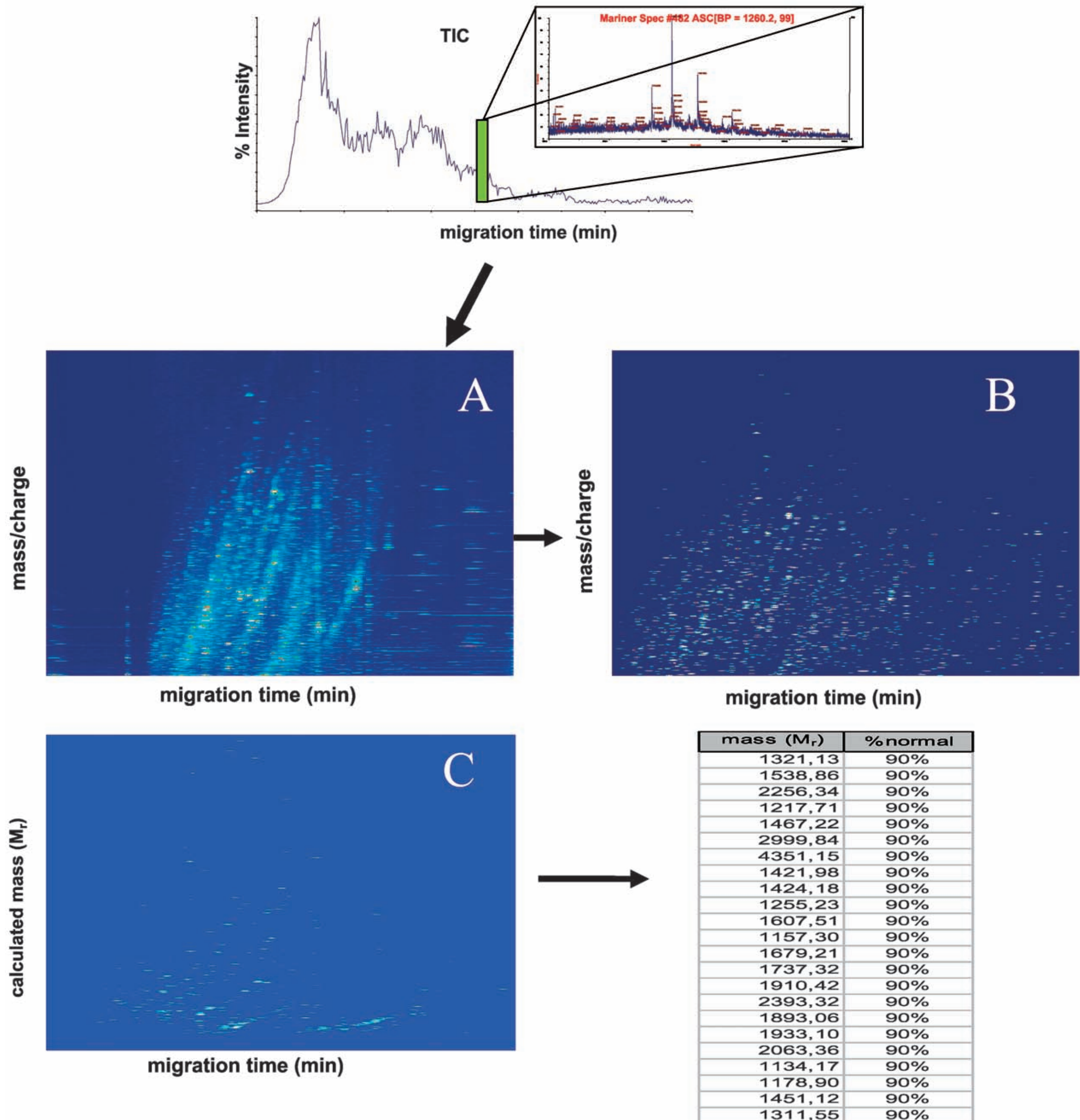
The detection algorithm performs a matched filtering on the raw data after adaptive baseline and noise-level estimation and hence is used to allocate charge, based on isotopic distribution. Rather than detecting single peaks (e.g., by threshold or slope) we match theoretical signal-envelopes to the spectral data. Matched filtering is well known for its superior performance in radar and communications engineering. Out of all possible digital filters, it yields maximum signal-to-noise ratios for detecting signals in the presence of noise. It is less sensitive to stray spikes, as it coherently integrates over the signal-envelope and measures the overall correlation between the data and theoretical model [25]. The major drawbacks of matched filtering include its computational expense and the necessity of a proper analytical model for the signals to be detected. In our case—the detection of polypeptide signatures—we are faced with yet another problem: the shape of the polypeptide signal depends on its mass, charge and the isotopic distribution of its constituents (e.g., poisson distributed). For a given charge, the envelope is fully described by its position on the mass per charge (m/z) axis. Hence, to detect polypeptides in any possible charge state, separate filters for each charge to be identified have to be applied. This results in a multitude of potential polypeptides in various charge states, only few of which are actually correct. Additionally, due to the periodicity of the isotopic envelopes, the filter response shows some form of aliasing, i.e., additional peaks adjacent to the actual polypeptide position. It is important to note that no signal is lost at this point, but the raw data is reduced to several hundreds of potential polypeptide locations.

In order to discard false and aliased peaks, all candidates are sorted by their significance and then successively subtracted from the raw data. Whenever this subtraction yields a reduction in signal magnitude, the peak is denoted as valid and stored.

3.2.2. Mass detection and charge conjugation

As described above, the isotopic structure of polypeptides is identified in the primary peak detection, as far as device resolution allows (in the case of the Mariner TOF instrument used, up to a charge of 6). Thus, a unique mass is assigned to the identified polypeptides. A substantial contingent of peaks however does not lend itself to this straightforward approach, as the charge of most of the larger polypeptides is too high to reveal their isotopic structure.

We employ a probabilistic clustering algorithm [26,27] to map charge-conjugated peaks onto ideal polypeptides of common mass and unit charge. In general, finding the underlying mass-defined polypeptides from an incomplete set of detected peaks is



mass (M_r)	%normal
1321,13	90%
1538,86	90%
2256,34	90%
1217,71	90%
1467,22	90%
2999,84	90%
4351,15	90%
1421,98	90%
1424,18	90%
1255,23	90%
1607,51	90%
1157,30	90%
1679,21	90%
1737,32	90%
1910,42	90%
2393,32	90%
1893,06	90%
1933,10	90%
2063,36	90%
1134,17	90%
1178,90	90%
1451,12	90%
1311,55	90%

Fig. 2. The MosaiquesVisu software allows the depiction of the information from a crude CE–MS analysis (upper part) as a three-dimensional contour plot (A). Here a contour plot of urine from a healthy volunteer is shown, mass per charge on the *y*-axis against the migration time in min (*x*-axis), signal intensity color coded. Next, the signal to noise is calculated and the noise removed, thus leaving only actual signals (B). The software calculates the actual mass (C) based on both isotopic distribution and conjugated masses. This leads to a table of up to 1000 polypeptides defined via their mass. As an example, the bottom right shows 23 polypeptides found in more than 90% of the healthy individuals analyzed.

not a trivial task and may often reveal more than one unique solution. Each of the experimentally observed CE–MS peaks (usually>1000) may carry an arbitrary charge and thus give rise to several potentially existing polypeptides of distinct mass. The clustering algorithm developed attempts to find a solution to this ill-defined optimization problem by iteratively associating each CE–MS peak with possible polypeptides and evaluating the probability for these polypeptides to exist.

Given a set of peaks, the probability of these originating from one common polypeptide depends on their m/z-location, migration time and relative abundance. Limiting ourselves to a maximal charge of 40, each peak may belong to one of up to 40 000 polypeptides. Thus, for a protein the exact mass, charge distribution and migration time, depends on its constituents (which in turn depend on the yet unknown protein parameters).

By applying these algorithms, the raw data from a typical CE–MS spectrum can be processed and a peak list can be calculated within less than 3 min.

3.3. Examination of urine

The application of proteomics for diagnostic purposes requires the establishment of a ‘‘normal’’ polypeptide pattern. Common technologies are surface-enhanced laser desorption ionization (SELDI) MS [28–31], high-performance liquid chromatography (HPLC)–MS [32–35] or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [e.g., off-line coupling with matrix-assisted laser desorption ionization (MALDI) MS] [4–6,16,36–38].

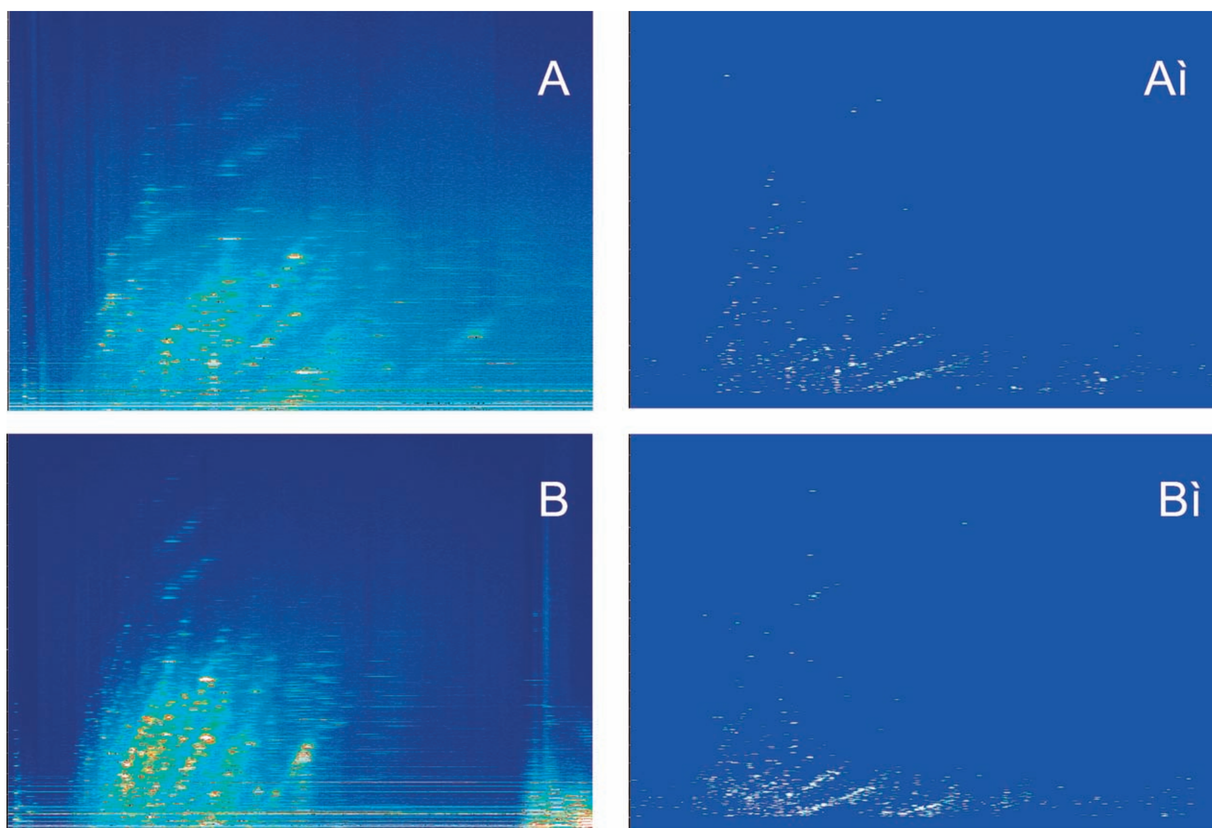


Fig. 3. Three-dimensional plots of CE–MS spectra of urine (A, B) from two different healthy volunteers. As evident, the overall peptide and protein pattern (Aì, Bì) observed is similar and easily comparable in all cases.

SELDI-MS is well suited for high sample throughput but the limitations in resolution lead to a loss of several proteins and peptides in the sample. This in turn leads to the establishment of rather low-resolution patterns, which represent only a minority of proteins and peptides present. Petricoin et al. [39] used this method to determine protein patterns in serum to identify ovarian cancer.

For HPLC–MS of human body fluids it is necessary to use tryptic digests during the preparation [33–35], since native proteins with higher relative molecular masses cannot be investigated. Spahr et al. [33] used this method and identified more than 100 polypeptides present in urine, but could not obtain total protein patterns.

Finally, 2D-PAGE is often used for protein separation in human body fluids [40] and in off-line combination with a mass spectrometer (mostly MALDI-MS), up to 1000 individual polypeptide spots can be identified, unfortunately in a very time consuming process. Thus the determination of a polypeptide pattern in a single, time-limited step would be a significant improvement.

For this purpose, urine from 18 healthy volunteers was collected, prepared as described and examined using CE–MS. Two typical three-dimensional CE–MS chromatograms (raw data contour plots A, B; three-dimensional protein–peptide plots A″, B″) of urine from healthy volunteers are shown in Fig. 3. The individual CE–MS runs with urine samples obtained from different volunteers revealed similar and highly comparable results. This enabled us to establish a ‘‘normal’’ pattern of polypeptides found in urine. To this end the individual CE–MS runs were analyzed with the mosaiquesvisu software and the polypeptide data were stored in an MS-Access database. Analysis of these data resulted in a list of 247 polypeptides, each of which present in more than 50% of the individual samples (Table 1), establishing the ‘‘normal urinary polypeptide pattern’’.

To evaluate the feasibility of the application of CE–MS for diagnostic purposes, urine samples from five patients with renal diseases and impaired renal function were examined. As shown in Fig. 4, the obtained urinary polypeptide patterns (C″, D″) were easily distinguishable from the patterns obtained from samples of healthy volunteers. Actual masses were calculated as described and compared within our database. As already expected from visual examination of the contour plots, several additional polypeptides were present in the patient samples. Twenty-seven additional polypeptides not found in

Table 1
Molecular masses of 247 polypeptides found in more than >50% of the healthy individuals

1028.50	1451.65	1766.02	2174.73	2742.18	3685.85
1046.59	1467.70	1776.70	2187.98	2752.58	3722.48
1047.50	1473.53	1779.51	2190.08	2761.35	3840.65
1050.64	1474.67	1782.26	2196.43	2769.62	3946.92
1075.55	1489.43	1822.32	2205.03	2787.66	3969.35
1099.56	1490.51	1824.43	2210.96	2800.01	3986.92
1110.35	1491.68	1829.13	2220.95	2808.65	4025.83
1122.46	1506.25	1840.54	2226.91	2823.22	4044.50
1125.48	1508.19	1849.44	2238.25	2825.19	4098.67
1134.57	1517.57	1854.75	2248.88	2849.11	4102.37
1139.61	1518.58	1863.75	2256.80	2854.07	4218.50
1141.52	1523.35	1873.05	2265.50	2861.89	4290.35
1147.46	1531.61	1874.33	2272.88	2864.17	4353.42
1157.56	1539.39	1876.44	2278.81	2889.47	4748.22
1160.50	1561.68	1878.29	2281.98	2977.50	4800.97
1179.43	1567.11	1879.67	2314.02	2986.15	5001.10
1194.50	1573.95	1885.67	2326.79	3001.94	5801.75
1195.38	1576.50	1889.78	2342.93	3011.22	6170.88
1209.33	1580.83	1893.06	2367.11	3013.14	6186.90
1224.60	1588.57	1894.93	2377.50	3018.43	6237.91
1235.26	1589.60	1910.95	2385.20	3021.29	9868.69
1239.38	1591.68	1933.52	2394.25	3023.62	12 718.30
1250.56	1594.58	1942.59	2406.76	3041.03	
1255.50	1609.72	1948.38	2409.88	3063.55	
1261.51	161 169	1954.74	2427.10	3082.25	
1265.57	1627.64	1965.30	2442.38	3091.88	
1283.43	1631.60	1977.10	2525.36	3098.68	
1286.05	1646.87	1989.46	2548.35	3109.17	
1297.51	1651.69	2007.74	2552.75	3121.07	
1306.53	1653.59	2010.50	2556.67	3136.86	
1321.55	1659.43	2020.02	2563.47	3149.52	
1322.64	1664.71	2025.51	2583.85	3152.56	
1343.41	1667.30	2038.92	2586.67	3166.09	
1351.59	1673.66	2046.66	2602.64	3208.73	
1366.63	1680.20	2048.06	2621.64	3265.27	
1368.72	1698.26	2058.97	2636.53	3271.30	
1378.59	1706.73	2063.93	2643.20	3280.93	
1389.67	1716.23	2069.95	2649.55	3290.71	
1422.50	1717.66	2080.81	2658.03	3295.56	
1424.26	1722.87	2104.21	2663.68	3343.29	
1425.56	1733.83	2112.92	2679.12	3385.72	
1433.55	1737.83	2117.00	2682.23	3402.08	
1435.57	1739.82	2153.96	2686.80	3458.18	
1438.57	1745.69	2158.82	2695.20	3495.63	
1446.56	1761.79	2169.57	2717.32	3531.09	

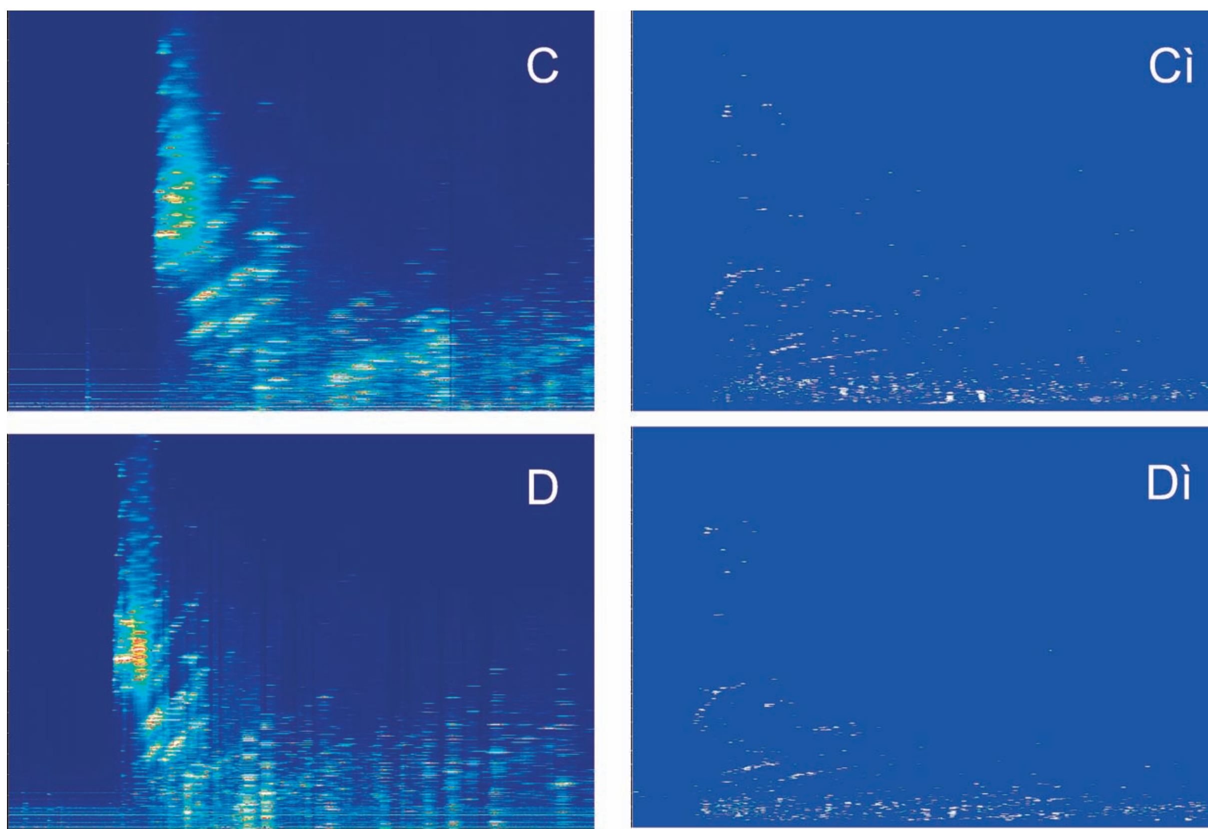


Fig. 4. Three-dimensional plots of CE–MS spectra of urine (C, D) from two patients suffering from membranous glomerulonephritis (MNGN). As evident, the overall peptide and protein pattern (Cì, Dì) observed again is similar in both cases, but distinctly different from the normal control. Several polypeptides present in these samples were not detected in the samples from the healthy volunteers.

the normal urinary polypeptide pattern appeared in more than 50% of the samples, while 13 of the ''normal polypeptides'' were absent in the patient samples. Since the protein content of the patient samples was much higher than that in the normal controls, the lack of certain polypeptides actually might be the result of deficient renal secretion or re-absorption. Although these data are obtained from a rather small number of patients, they indicate that evaluation of proteomic patterns in urine might serve as a diagnostic tool to further characterize diseases.

Taken together, we have demonstrated that it is possible to analyze, in one single, time-limited step, an exhaustive polypeptide profile of human urine. The technique used allows to analyze up to 2000 polypeptides simultaneously and to interpret the raw data.

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