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Journal of Chromatography A, 1027 (2004) 203–212
JOURNAL OF CHROMATOGRAPHY A
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Micellar electrokinetic capillary chromatography and data alignment analysis: a new tool in urine profiling

Christelle Guillo [a], David Barlow [a], David Perrett [b], Melissa Hanna-Brown [a,*]

[a] *Department of Pharmacy, King's College London, London SE1 9NN, UK*
[b] *Department of Medicine, Bart's and The London School of Medicine and Dentistry, London EC1A, UK*

Abstract

The complex nature of biofluids demands efficient, sensitive and high-resolution analytical methodologies to examine how the 'metabolic fingerprint' changes during disease. This paper describes how sulphated β-cyclodextrin-modified micellar electrokinetic capillary chromatography (SβCD-MECC) has been combined with data alignment analysis and may prove a useful new tool in urine profiling, allowing for separation of over 80 urinary analytes in under 25 min. The optimised and validated SβCD-MECC methodology combined with data alignment analysis provides rapid identification of 'mismatches' between urine profiles which are not easily detected with the naked eye as well as a 'similarity score' which indicates the total sum of differences between one profile and another. The combination of SβCD-MECC with data alignment software should prove a useful alternative tool in metabonomic studies for rapid comparison of urine profiles.

Keywords: Electrokinetic chromatography; Data alignment; Cyclodextrins

1. Introduction

Recently there has been a fresh focus on metabonomic approaches for quantifying metabolite levels in whole organisms. For example a urine sample can potentially yield a plethora of information related to thousands of compounds, such as inorganic ions, organic acids, amino acids, purines, pyrimidines, etc., resulting from endogenous or exogenous metabolic pathways. The accumulation of characteristic metabolites in urine (biomarkers) is specific to many diseases. The semi-non-invasive nature of sampling has prompted researchers to develop urinary profiling methods which may be of value in a clinical setting for disease diagnosis or monitoring disease progress.

To these ends, metabonomic approaches using diverse analytical probes, including chromatographic and spectroscopic techniques have been documented. Urine profiling studies were reported in the 1950's using paper and ion-exchange chromatography to analyse amino acids [1]. Later, two-dimensional thin layer electrophoresis and chromatography were used successfully to screen for amino acid disorders [2] and inborn errors of purine and pyrimidine metabolism [3,4]. Coupled anion and cation exchange chromatography was also utilised to separate over 100 UV-absorbing constituents in urine [5]. More recent chromatographic and hyphenated chromatographic-spectroscopic techniques, e.g. gas chromatography (GC) and GC–mass spectrometry (MS) methods have been used in screening for biomarkers [6–15], and in drug metabolism studies [16]. Similarly, high-performance liquid chromatography (HPLC) and HPLC–MS have proven useful tools in diagnosis of disease [17–19] and for profiling of metabolites in urine [20–22].

Spectroscopic techniques have also been extensively used in metabonomic studies, in particular NMR which has proven extremely useful not only for profiling urine but also in identification of a host of both endogenous and exogenous biomarkers of various diseases [23–31]. In contrast, the reports documenting MS screening are far more limited [32,33]. However, all of the techniques described above usually require tedious sample preparation (including extraction and/or derivatization, etc.) resulting in total analysis times being excessively long. For this reason, recently, capillary electrophoresis (CE) has received more attention in its capacity as a new and more powerful analytical tool for urine profiling, due to its advantageous characteristics

* Corresponding author. Tel.: +44-207-848-4722; fax: +44-207-848-4800.
E-mail address: melissa.hanna-brown@kcl.ac.uk (M. Hanna-Brown).

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doi:10.1016/j.chroma.2003.11.102

of enabling highly efficient separations of diverse components present in minute sample volumes. Thus, over recent years reports have emerged detailing the successful application of CE and CE–MS in diagnosis of disease [34–46].

One common feature within the metabonomics arena, and even within many of the reports mentioned, is that some means of discrimination between complex profiles is an important requirement. Chemometric approaches commonly referred to as pattern recognition (PR) techniques have therefore been an area of particular focus to aid the close examination of changes in the 'metabolic fingerprint'. Such approaches however allow for predictive models to be constructed using a 'training set' of profiles allowing diagnoses from subsequent sample data. These chemometric approaches allow for detection of variations in levels of low concentration metabolites which can be difficult to detect with the naked eye, yet are often important biomarkers indicating a metabolic defect. While pattern recognition has been extensively used for urinary profiling post NMR, GC and to a lesser extent HPLC analyses, its use in combination with CE has not yet been fully exploited. Indeed, the lack of reproducibility inherently observed in CE analyses is probably to blame for conventional PR methods not being extensively used for whole CE profiles. The literature contains reference to techniques by which electropherograms may be 'normalised' in order to improve reproducibility between repeat analyses using for example the current output or reference peaks. In the former method, migration time shifts can sometimes be explained (at least in capillary zone electrophoresis (CZE)) by current variations, wherein migration times can be simply multiplied or divided by current values [47] and variations in electroosmotic flow (EOF) can hence be more or less eliminated and reproducibility improved. However, in micellar electrokinetic capillary chromatography (MECC), simply dividing or multiplying the migration time by the current value is inadequate to explain all of the variations occurring in the EOF. This is understandable, as the factors contributing to EOF variation in complex MECC systems are numerous. For example, the polydispersity of micelles can change from one run to another as may the relative amounts of additives which may affect the interactions. In the latter method reference peaks are selected, preferably one at the beginning and one at the end of the electropherogram, and the migration times of these two peaks are used to correct for migration time shifts in the electropherograms under investigation [48]. However, this method again while being suitable for simpler systems (CZE) is not always useful for complex systems where EOF variations have numerous contributing factors. In our investigations we have tried both of these methods without success, but were convinced of the necessity of improving electropherogram reproducibility. This problem is also common to other forms of data alignment where there is redundant information, as for example in alignment of DNA sequences. In this paper we have adapted one form of DNA sequence alignment to the problem of drift in MECC where baseline is inserted or erased when shifts occur across the electropherogram, hence removing variations due to the EOF.

The aims of this study were therefore (i) to investigate the utility of CE as a metabonomic tool in urinary profiling, with particular attention to the versatile MECC mode of CE and (ii) to investigate the use of complete MECC electropherogram, together with data alignment methodology, in the development of clinically useful predictive models.

2. Experimental

2.1. Chemicals

Sodium borate, sodium dodecyl sulphate (SDS), sulphated β-cyclodextrin (SβCD) and EDTA were purchased from Sigma (Poole, UK) and were all analytical grade. Sodium hydroxide and hydrochloric acid were purchased from BDH (Poole, UK). Sodium metabisulfite was obtained from Thornton & Ross (Huddersfield, UK). Reverse osmosed deionised water (Milli-Q Synthesis from Millipore, Bedford, MA, USA) was used for standard solution and electrolyte preparations.

2.2. Urine specimens and sample preparation

Human urine samples collected from the same volunteer before and 2 h after ingestion of a 500 mg paracetamol tablet, were used in 'proof of principle' studies. Stability investigations were performed on a pooled human urine sample which was prepared by combining 18 early morning samples collected from human volunteers.

Before analysis, samples were allowed to equilibrate to room temperature before use and then vigorously shaken for approximately 1 min before filtration through a 45 μm filter (Whatman, Clifton, NJ, USA).

2.3. Instrumentation

CE experiments were carried out on a P/ACE capillary electrophoresis system 5510 or a P/ACE MDQ system (Beckman Instruments, Fullerton, CA, USA) fitted with a diode array UV-absorbance detection (190–600 nm), a temperature-controlled capillary compartment (liquid cooled) and an autosampler. Electrophoretic data were acquired and analysed with the P/ACE station (P/ACE 5510 instrument) and 32 Karat software (P/ACE MDQ instrument). Separations were performed in a fused silica capillary (50 μm i.d.) (Composite Metal Services, Hallow, Worchester, UK). New capillaries were conditioned for 30 min at 25 °C with 1 M NaOH, followed by 0.1 M NaOH for 20 min and deionised water for 10 min. The capillary was washed with 0.1 M NaOH and deionised water for 1 min, and then 2 min with the run buffer before each analysis.

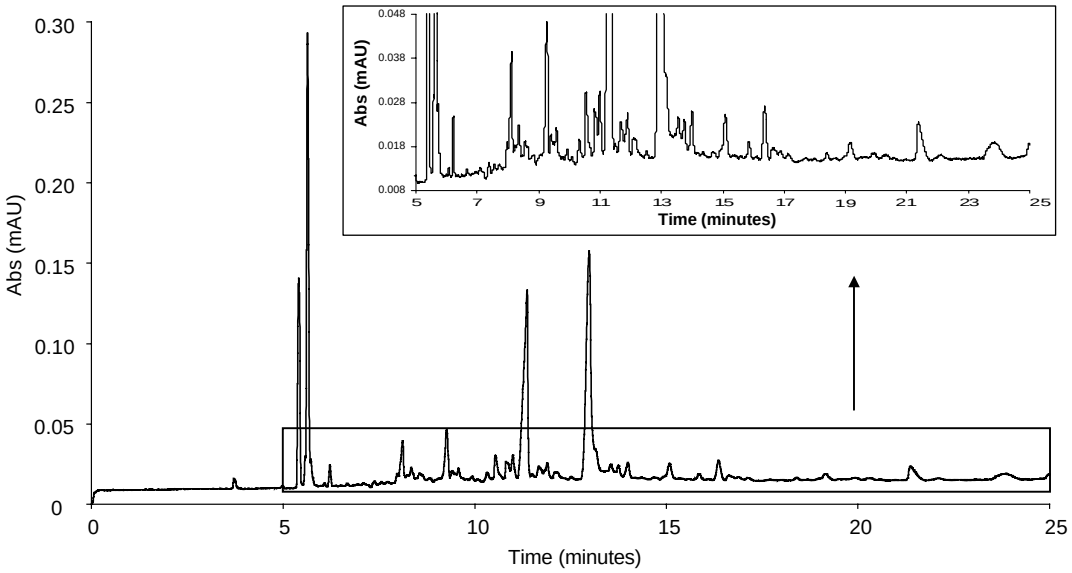

Fig. 1. Typical electropherogram produced from the SβCD-MECC analysis of a human urine sample (expanded region also shown between 5 and 25 min). Conditions: sodium borate/SDS/sulphated β-cyclodextrin (25/75/6.25 mM), pH 9.50; 18 kV, 20 °C, 5 s injection at 0.5 p.s.i. (1 p.s.i. = 6894.76 pa); detection at 200 nm.

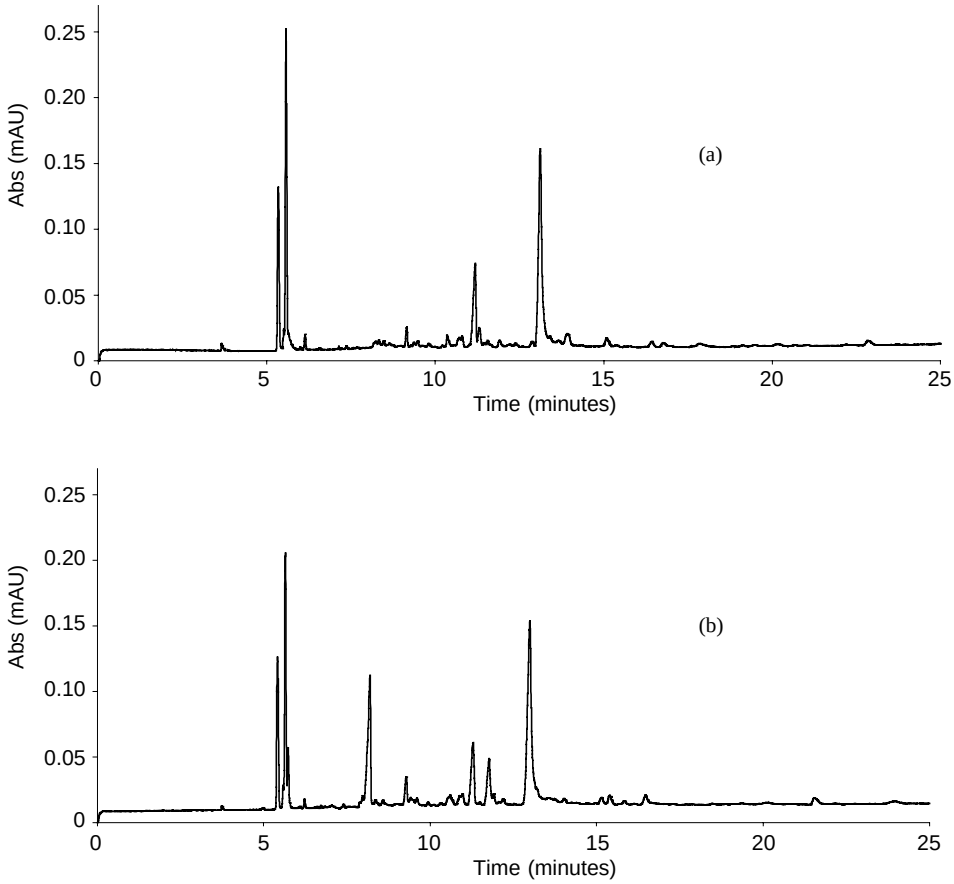

Fig. 2. Electropherograms of (a) a blank urine sample and (b) a urine sample collected 2 h after ingestion of a 500 mg paracetamol tablet prior to data alignment. Samples were analysed using the SβCD-MECC methodology as described in Fig. 1.

2.4. Sulphated β-cyclodextrin-modified MECC methodology

The SβCD-MECC method for urine analysis by Alfazema et al. [49] was further optimised and validated (details to be published shortly [50]). The running buffer was composed of 25 mM sodium borate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin. The pH was adjusted to pH 9.50 with 1 M NaOH, after addition of SDS and cyclodextrin. Buffer solutions were filtered through a 45 μm filter before use. Optimum conditions for urine analysis were obtained using a 47 cm capillary (40 cm effective length) maintained at 20 °C, 18 kV applied voltage and 5 s hydrodynamic injection.

2.5. Data alignment

CE data were exported as an ASCII file and treated as a UV absorbance/time series, comprising a total of (N) 6000 data points. In order to correct for base line noise and drift, the electropherograms were first convoluted with a simple sliding ramp function, with absorbances recorded at time-point n (A_n) transformed as:

$$A'_n = \sum_{k=-2}^{k=2} kA_{n+k}$$

with n varied from 3 to N-2. The resulting first derivatives of the profiles were then mapped as ASCII charac-

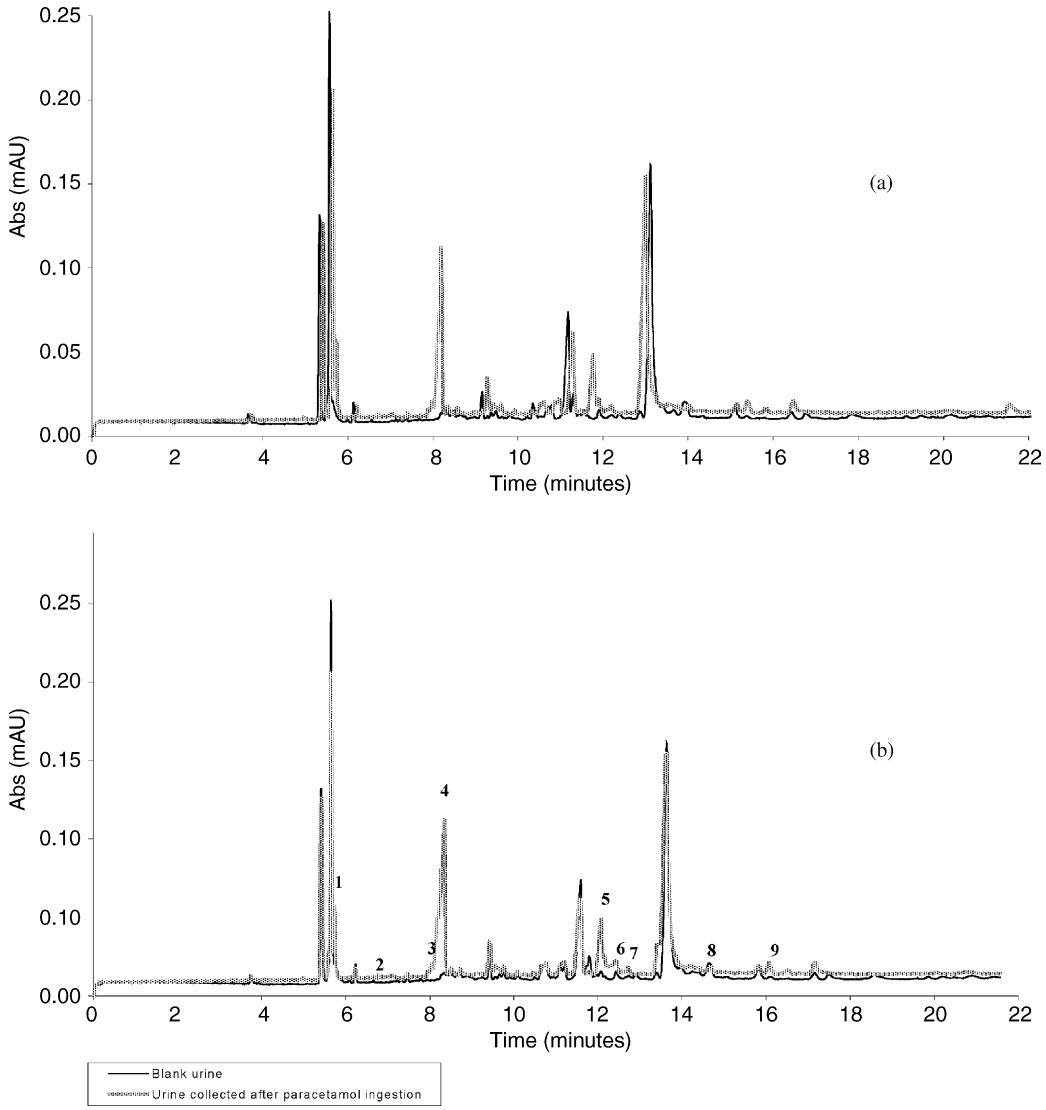

Fig. 3. Comparison of the urine profiles of blank urine and urine collected 2 h after paracetamol ingestion (a) before and (b) after pair-wise data alignment analysis. Numbered peaks represent new peaks or changes in analyte levels detected in the paracetamol urine profile compared with a blank urine. Peak identifications are: 1 = creatine, 2, 3, 6–9 = unknown analytes, 5, 6 = paracetamol metabolites (refer to corresponding data alignment report displayed in Table 1).

ter strings, with: $-0.001 < A_i < 0.001$ coded as 'N', $A_i < -0.001$ as 'M' and $A_i > 0.001$ as 'P'. All sequences of the form $P_5N_iM_5$, with $0 \leq i \leq 6$, were then re-coded so as to flag the crowns of peaks in the profiles as 'L'; the sequence P_5NNM_5, for example, thus re-coded as P_5LLM_5.

Pair-wise alignments of the electropherogram character strings were performed using the dynamic programming algorithm of Needleman and Wunsch [51], as implemented in the SEQSEE package [52]. Self-matches between the characters 'M' and 'P' were each scored 10, and the self-matches between the characters 'N' and 'L' were scored as 0 and 50, respectively. Mismatches of 'N' against any other character were scored zero, and those involving any pair of 'P', 'M' or 'L' were penalised by scores of −10. No penalty was incurred for any gap *creation*, but gap *extension* was penalised by a score of −5 for each gap position inserted. All data alignment programs were operated via a standard personal computer.

3. Results and discussion

3.1. Proof of principle studies

As a 'proof of principle', urine profiles obtained using our optimised SβCD-MECC method, (where over 80 compounds in urine can be separated in under 25 min, Fig. 1) were subjected to data alignment procedures. In the first instance, urine profiles obtained from a blank ('normal') urine sample and a urine sample collected after ingestion of a paracetamol tablet were used to demonstrate the principle of data alignment analysis, since they appeared to be significantly different (by eye) (Fig. 2).

Table 1
Pair-wise alignment analysis report of blank urine and urine collected 2 h after paracetamol ingestion

Electropherogram data produced from blank urine		Electropherogram data produced from urine collected 2 h after paracetamol ingestion	
Peak migration time (min)	Peak area	Peak migration time (min)	Peak area
3.65	0.307	3.73	0.153
		4.98	0.126
5.33	2.510	5.41	2.647
5.56	4.041	5.65	2.679
		5.73[1]	1.408
5.99	0.125	Not detected	
6.13	0.279	6.22	0.229
		6.71[2]	0.159
7.13	0.087	Not detected	
7.36	0.134	7.40	0.153
		7.97[3]	0.183
8.24	0.163	8.19[4]	2.352
8.33	0.223	8.35	0.194
8.47	0.214	8.57	0.272
9.15	0.368	9.27	0.656
9.27	0.109	9.41	0.172
9.36	0.141	Not detected	
9.47	0.239	9.60	0.299
9.80	0.197	9.93	0.172
10.21	0.118	Not detected	
10.35	0.452	10.6	0.667
10.71	0.177	Not detected	
10.79	0.378	10.96	0.481
11.18	1.030	11.28	1.408
11.30	0.525	11.49	0.185
11.44	0.125	Not detected	
11.55	0.204	11.77[5]	1.070
11.90	0.282	11.90[6]	0.421
		12.18[7]	0.325
12.87	0.267	Not detected	
13.11	8.713	12.99	7.382
13.40	0.455	Not detected	
		14.02[8]	0.367
15.09	0.538	15.14	0.402
		15.39[9]	0.559
16.42	0.350	16.46	0.528

Numbered peaks represent new peaks or changes in analyte levels detected in the paracetamol urine profile compared with a blank urine. Peak identifications are: 1 = creatine, 2, 3, 6–9 = unknown analytes, 5, 6 = paracetamol metabolites (refer to corresponding electropherograms shown in Fig. 3b).

The electrophoretic data were subjected to pair-wise alignment analysis as described in the Section 2. In each analysis, the electropherograms compared are aligned and a report is provided listing peak migration times and integrated areas, giving additional indications of unique peaks and peak equivalences. Fig. 3 and corresponding data displayed in Table 1 demonstrate how the peaks common to both blank and post-paracetamol urine profiles were aligned, revealing the presence of new peaks in the electropherogram obtained from urine collected after paracetamol

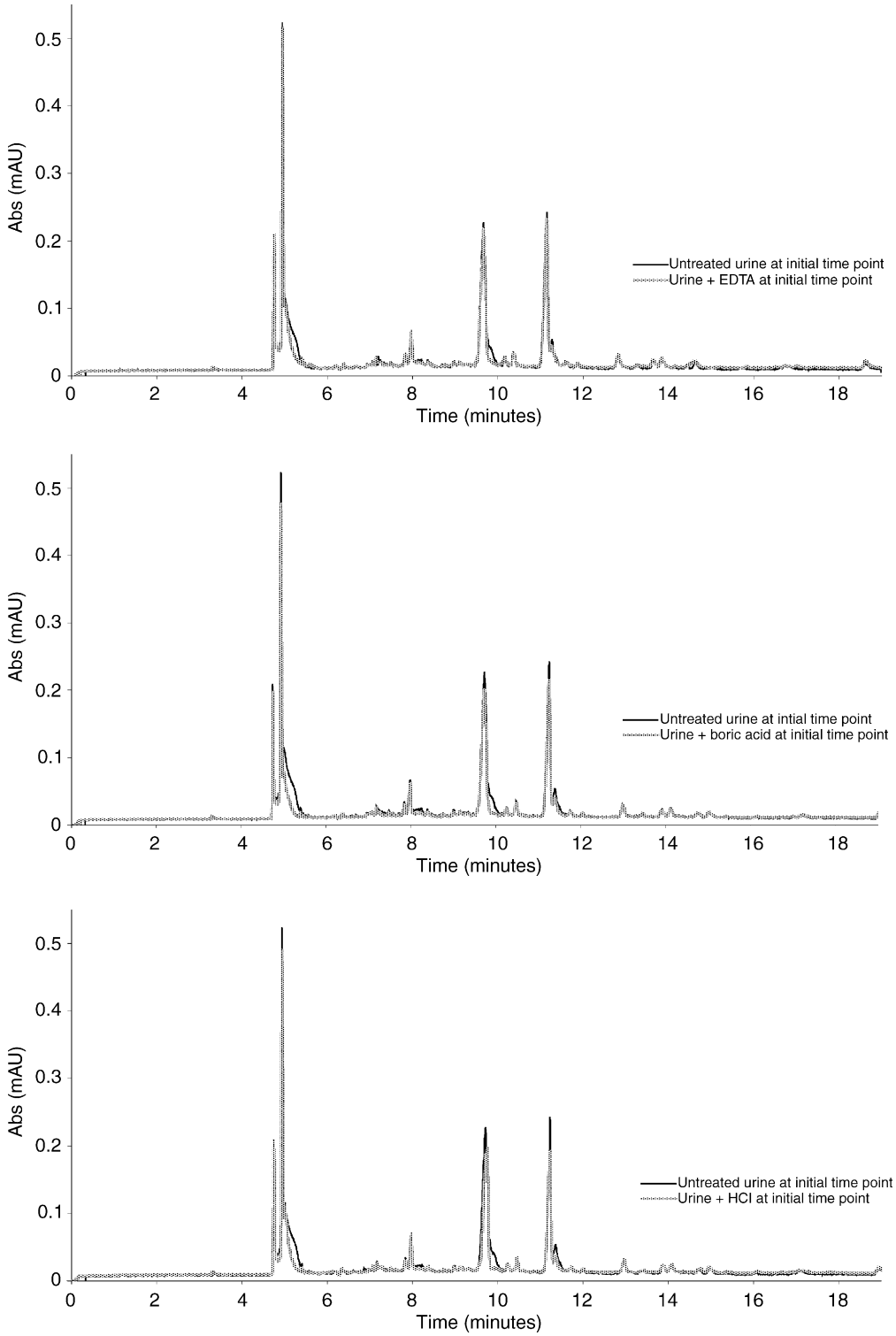

Fig. 4. Aligned electropherograms of treated urine (with EDTA + sodium metabisulfite, boric acid, HCl) with untreated urine at initial time-point.

ingestion. The corresponding alignment report (Table 1) indicates that 15 peaks were detected to be 'different', with 7 new peaks appearing in the post-paracetamol urine profile.

This exercise indicates that the data alignment program allows for the detection of changes in profiles, including extra and/or disappearing peaks and variations in analyte levels, confirming its ability to provide a rapid and efficient comparison between two electropherograms.

3.2. Application to a stability study

This procedure was repeated in a similar manner to a stability study where more subtle differences in the profiles were expected. A large set of sample profiles, produced during an investigation where preservatives and storage methods for urine, were assessed. Urine samples were pre-treated in one of the following ways: (1) acidification with HCl, (2) addition of boric acid, (3) addition of EDTA and sodium metabisulfite (preservative and antioxidant respectively) and (4) untreated. These samples were then stored in different conditions for 14 months including: (a) fridge at 4 °C, (b) freezer at −20 °C and (c) freezer at −80 °C. The profiles of these stability samples were investigated immediately after treatment (initial time-point) and then at various intervals up to 14 months. The initial and end-point (14-month) data sets of treated and untreated urine samples were selected for pair-wise alignment analysis since their electrophoretic profiles appeared to have changed by eye under the different treatments and storage conditions over time.

The effect of each treatment on the overall urine profile was assessed using pair-wise data alignment analysis. Firstly, the effect of preservative on the urine profile at initial time-point was assessed by (i) examination of the aligned profiles (Fig. 4) and (ii) examination of the data alignment similarity score. The similarity score is an output from the data alignment analysis and sum of the absolute peak area between two profiles. The similarity score thus quantifies both the appearance and disappearance of peaks in profiles as well as peak areas arising from changes in analyte levels. The aligned electropherograms for each of the three samples treated with preservative overlaid on the initial untreated urine profile are shown in Fig. 4 where it is difficult to see significant changes in the profiles by eye. However, on examination of the similarity score from these alignments (Table 2) it can be seen that preservatives do in fact affect the profile, with boric acid and HCl acidification being the 'best' and 'worst' preservatives, respectively.

Table 2
Comparison of the effect of each treatment on the urine profile compared with untreated urine at initial time-point (similarity score)

Samples	Similarity score
Untreated urine vs. untreated urine	0.00
Urine + HCl vs. untreated urine	22.87
Urine + boric acid vs. untreated urine	8.13
Urine + EDTA with sodium metabisulfite vs. untreated urine	15.88

The data alignment methodology was next applied to compare urine profiles from the control and preservative treated samples at initial time-point with those after 14-month storage at 4, −20 and −80 °C. Results are shown in Fig. 5, where the similarity score between the urine at initial time-point and 14-month time-point are compared for each sample at each storage condition. These results would indicate again that the profile from urine acidified with boric acid is the

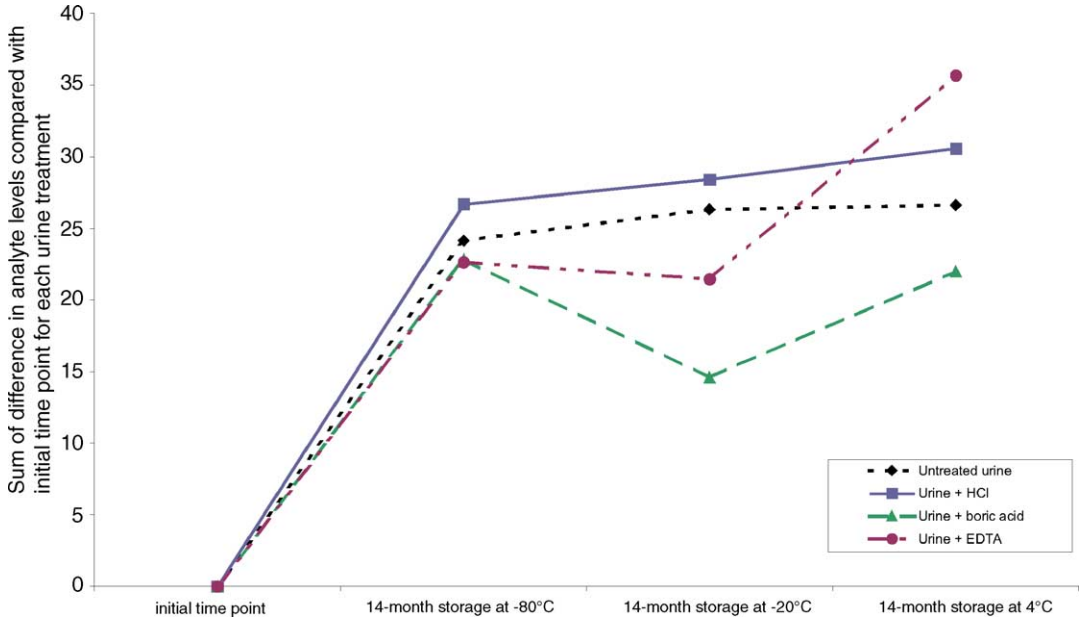

Fig. 5. Graph showing the changes in 'similarity score' of urine profiles from initial and 14 month time-point samples (untreated, HCl, boric acid, EDTA + sodium metabisulfite) stored under various conditions (4, −20 and −80 °C) when compared to their respective initial time-point profile.

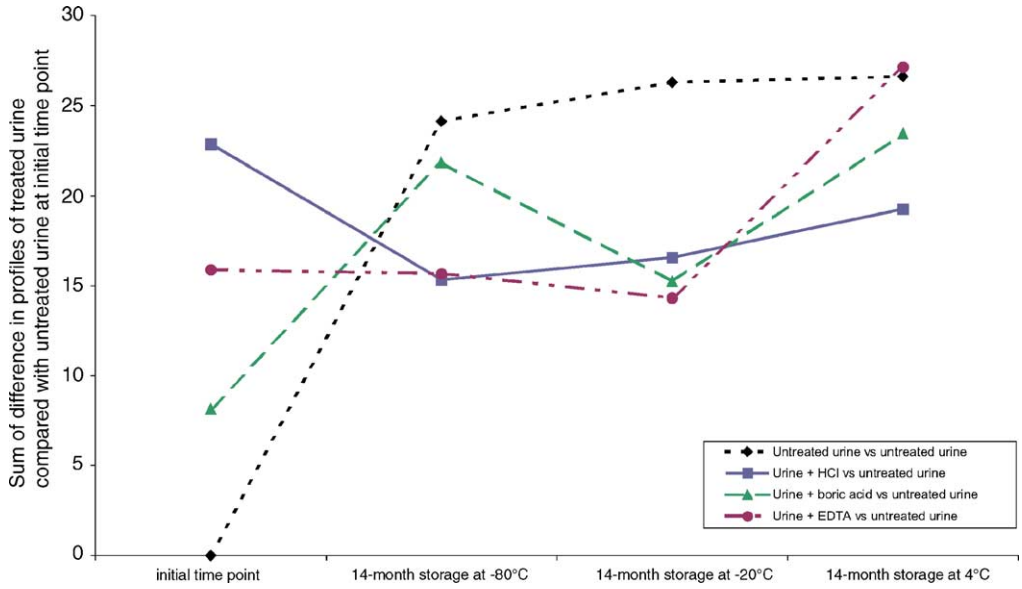

Fig. 6. Graph showing the changes in 'similarity score' of urine profiles from initial and 14 month time-point samples (untreated, HCl, boric acid, EDTA + sodium metabisulfite) stored under various conditions (4, −20 and −80 °C) when compared to untreated urine at initial time-point.

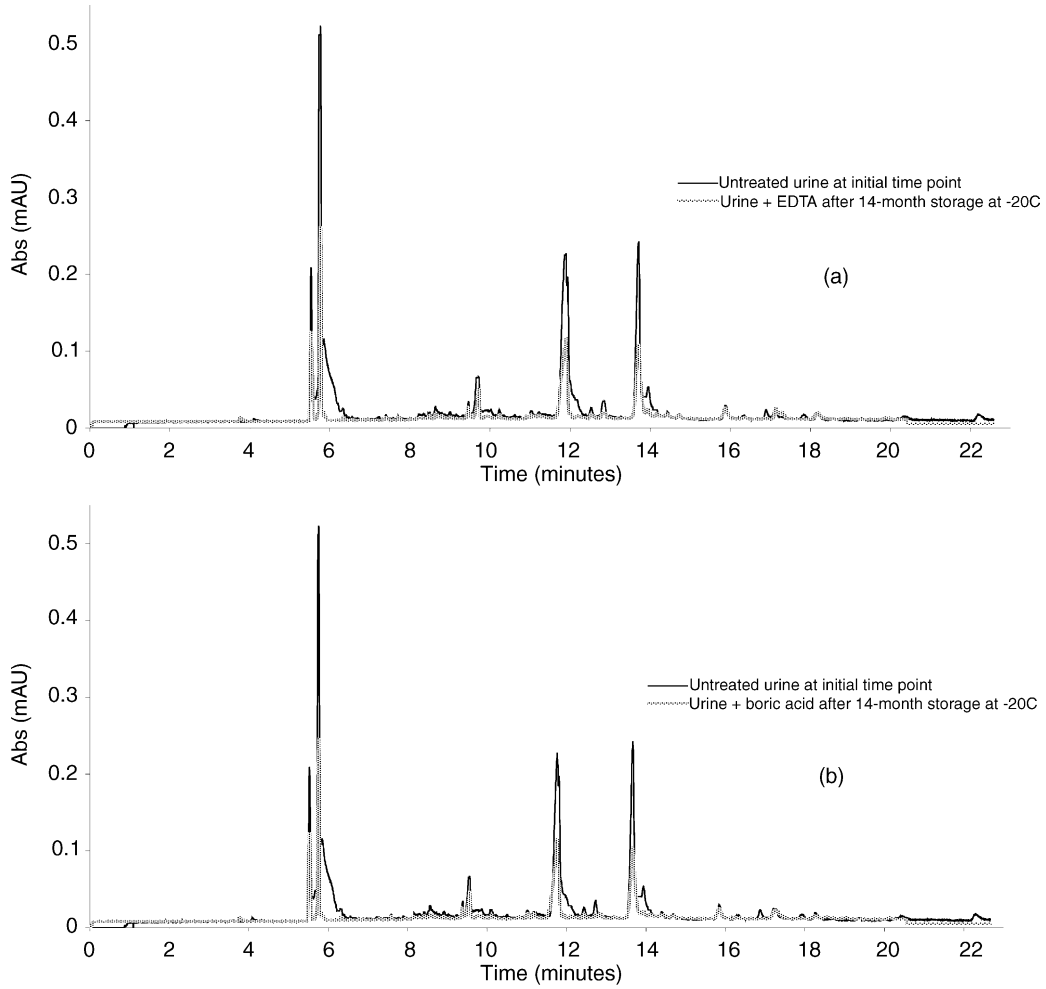

Fig. 7. Electropherograms of (a) urine + EDTA + sodium metabisulfite after 14-month storage at −20 °C and (b) urine + boric acid after 14-month storage at −20 °C, both aligned with untreated urine at initial time-point. These treatments/storage conditions appear to produce the best stability of urine over time.

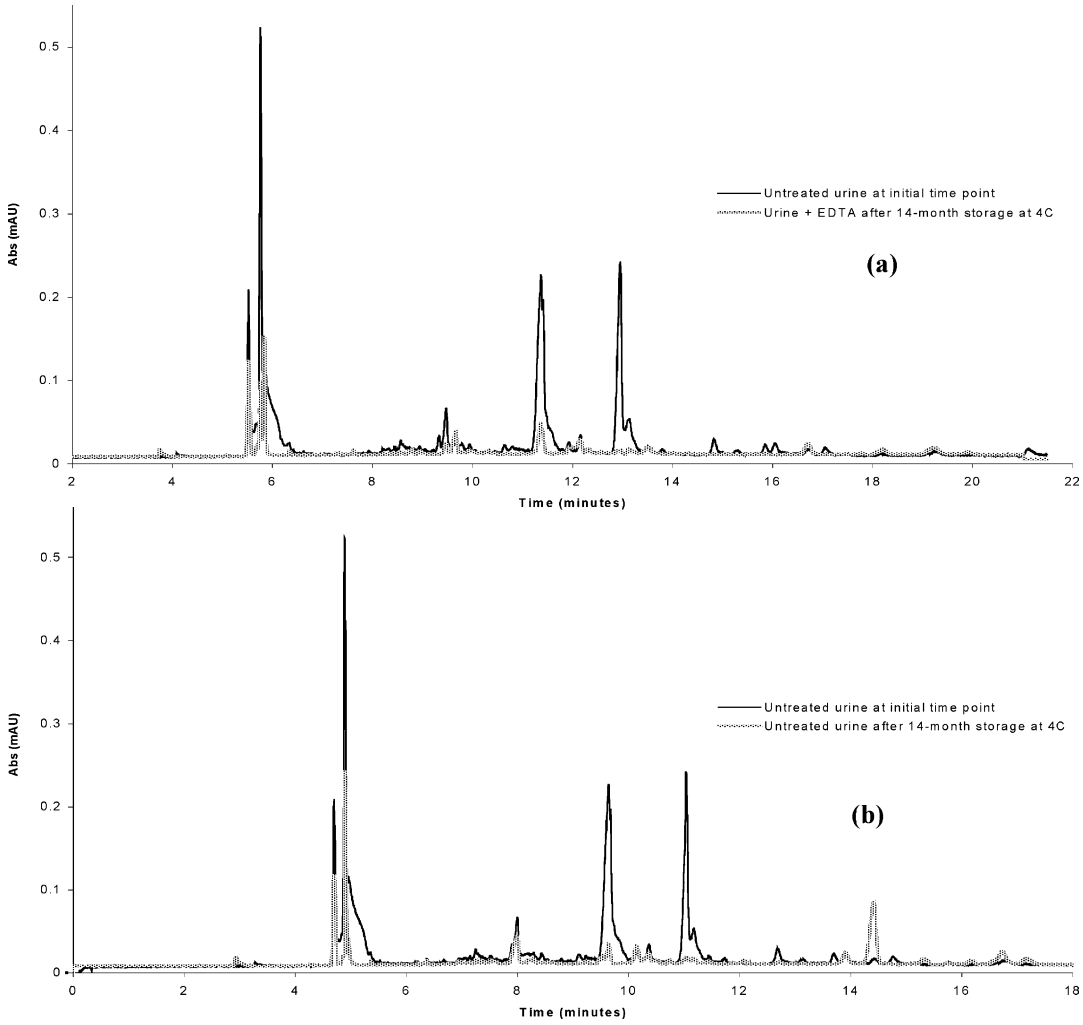

Fig. 8. Electropherograms of (a) urine + EDTA + sodium metabisulfite after 14-month storage at 4 °C and (b) untreated urine after 14-month storage at 4 °C, both aligned with untreated urine at initial time-point. Urine stability appears to be the worst using these treatments/storage conditions.

most 'similar' to that obtained at initial time-point, with −20 °C being the best storage condition.

Finally, the urine profiles for each sample under each storage condition were compared with the untreated urine (Fig. 6) in order to ascertain the effect of preservative on urine stability over time. Results shown in Fig. 6 reveal that although preservatives do affect the initial urine profile differently, the similarity score is generally lower when samples are either acidified with boric acid or treated with EDTA and sodium metabisulfite and stored at −20 and −80 °C, respectively. The aligned electropherograms for one of the 'best' and 'worst' conditions are shown in Figs. 7 and 8, respectively. Fig. 7 shows the aligned profiles of untreated urine (initial time-point) and urine treated with EDTA and sodium metabisulfite (a) and urine acidified with boric acid (b) after 14-month storage at −20 °C. It can be seen that these profiles are not significantly different. However, Fig. 8 shows aligned electropherograms of untreated urine (initial time-point) compared with urine preserved with EDTA and sodium metabisulfite (a) and non-treated urine (b) both stored at 4 °C. Here it is clear that the 4 °C storage condition is unsuitable, as profiles appear to be very different from the initial time-point profiles in each case. This confirms the fact that the profile alignment similarity score provides a rapid and reliable means by which an assessment can be made as to the 'similarity' of one profile to another without the need to examine the electropherograms closely. Such a procedure would be particularly advantageous should there be a large data set for comparison and/or pattern changes that are difficult to assess by eye (the latter situation arising for example when it may only be small changes in numerous analyte levels which are indicative of disease).

Finally, it may be noted that the pair-wise electropherogram alignments reported here can be readily elaborated to provide for comparisons between any number of electrophoretic profiles, using the same kinds of tools developed for comparisons involving multiple protein sequences (Clustal-W program [53]). With such multiple alignments, data from numerous MECC profiles corresponding to 'normal/control' samples could be used to produce a con-

sensus profile, against which any test profile could then be compared (by pair-wise alignment), and the resulting alignment and similarity score used to quantify 'abnormality'. Although the multiple alignment of profiles required for producing the consensus profile would be time-consuming, any subsequent consensus profile-test profile pair-wise alignment would be completed (as here) in a matter of seconds.

4. Conclusion

Although the sulphated β-cyclodextrin-modified MECC method developed for urine analysis is capable of high resolution, it is variable in terms of migration times and the 'normalisation' procedures found in the literature could not successfully correct this problem. However, a simple method adapted from genomic procedures was proven more powerful to correct for migration time shifts. This procedure has been demonstrated as a useful tool for aligning electropherograms as well as providing a 'similarity score' output, measuring differences in the profiles, and an aligned data output, highlighting areas in the electropherogram which have contributed to the differences observed in the profiles.

The combination of SβCD-MECC with data alignment analysis will thus provide an important new tool for metabonomic studies and prove invaluable in the rapid comparison of urine profiles and indication of metabolic disorders or abnormalities.

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