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# Potential of capillary electrophoresis, tandem mass spectrometry and coupled capillary electrophoresis–tandem mass spectrometry as diagnostic tools

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**Abstract**

Urine and blood samples from patients with known metabolic disorders have been analyzed by CE, MS–MS and CE–MS–MS. For the identification of defects in acylcarnitine metabolism, blood spots on filter paper were analyzed using an MS–MS ''neonatal screening'' approach. Direct CE–MS–MS analysis was used for the analysis of urine samples from patients with different metabolic disorders, including galactosemia, neuroblastoma, Zellweger syndrome, propionic acidemia and alcaptonuria. The sensitivity of the CE–MS–MS method was increased by use of multiple reaction monitoring.


*Keywords:* Metabolic disorders; Multiple reaction monitoring

## 1. Introduction

The development of new capillary electrophoresis–mass spectrometry (CE–MS) interfaces and the more rugged designs of MS–MS instruments over the last several years have made CE–MS–MS an interesting technique for the clinical laboratory where fast and accurate analysis is of major importance [1,2]. Both CE, being fast and simple, and MS–MS with its capability to identify compounds present in highly complex matrices, are techniques with a high potential and a promising future in the diagnostic laboratory.

### *1.1. Metabolic disorders*

Metabolic disorders are a class of rare diseases characterized by the accumulation of characteristic metabolites (diagnostic metabolites) in body fluids such as urine and blood. The diagnostic metabolites accumulate as a result of genetic defects causing decreased enzyme activity. The metabolites consist of a wide variety of chemical classes including steroids, carbohydrates, amino acids, polysaccharides, purines and pyrimidines, and organic acids. Combined with clinical information, the accurate identification of these metabolites can aid in the diagnosis of the disease [3–6]. Ideally, the diagnosis would be confirmed by measurement of the residual enzyme activity, or identification of causal DNA mutations. The quantity of characteristic metabolites, the residual activity of an enzyme, or the specific genotypes may also give information on the prog-

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nosis and severity of the disease. If diagnosed at an early stage, therapy such as a specialized diet or supplementation of missing metabolites (e.g., biotine, L-carnitine, vitamin $B_{12}$, etc.) may improve the course of the disease. This is of major importance as accumulation of metabolites to a toxic level can result in severe and irreversible damage to organs, especially the brain.

For the last 3 decades, gas chromatography (GC)–MS has been used routinely as a screening method for the analysis of patient urine for the diagnosis of metabolic disorders [7]. In most cases, the presence of abnormal metabolites can easily be revealed by visual inspection of the metabolite profile, and thus quantification is normally not necessary. When a deviation from the normal metabolite pattern is found, unknown compounds can be identified by comparing the MS spectra of the abnormal peaks with spectra of reference compounds in MS libraries. Two significant drawbacks of the GC–MS technique are the long time for sample preparation and analysis (about 3 h) and the limitations imposed by the requirement of sample volatility, which excludes many compound classes [8]. These drawbacks, typical of GC–MS analysis, have resulted in the need to find new, reliable and fast methods for screening patient samples for metabolic disorders.

With this in mind, we earlier developed a CE method using diode-array detection (DAD) with direct injection of urine samples [9]. Over 50 metabolites were separated in less than 15 min, and the diagnostic metabolites were identified by the comparison of the relative migration times and diode-array spectra with the same set of data for authentic compounds. Although over 25 disorders may be recognized with the CE–DAD technique, this approach is not applicable to metabolites which have weak or no UV chromophores. To overcome this limitation, the fast and simple CE methodology developed previously was combined with MS–MS which is a more universal and accurate detection method. This also generated enhancements in both sensitivity and specificity.

### *1.2. Single quadropole versus triple quadropole MS systems as detectors for CE*

Presently, there are a limited number of clinical CE–MS and CE–MS–MS applications described in the literature [10–16]. Therefore, it is felt that some basic principles of this technique should be discussed. A quadropole mass spectrometer consists of an ion source, one or more quadropoles (Q), and a multiplier (detector). At the inlet of the MS system, analytes of interest that are in a liquid phase (e.g., CE buffer) must be converted to gas phase ions. This is achieved in the ion source, where the liquid from the CE capillary is mixed with a coaxial sheath flow of a conducting liquid with high organic content. Outside this sheath flow, a nebulizing gas is used to improve the spray characteristics and enhance ionization. The metal tubing surrounding the CE capillary works as the grounding electrode, and the sheath liquid, which is in contact with both the CE effluent and this metal tubing, serves as the electrical contact. The high organic content (e.g., methanol or acetonitrile) of the sheath liquid makes the liquid forming the spray more volatile producing enhancements in sensitivity. The gas phase ions are produced by electrospray ionization (ESI) in which a voltage of 3 to 5 kV is applied to the nebulized liquid. Both positive and negative ions can be produced, and the mass analyzer can be used to detect one or both of these polarities in a single analysis.

The quadropole serves as a mass filter enabling the determination of the molecular mass of the analytes. In many instances, this gives sufficient information to identify the analytes of interest. The situation becomes more complicated with complex matrices such as urine where several compounds with the same molecular mass may be present. This does not present a problem if the compounds can be separated prior to entering the MS analyzer. Although CE possesses tremendous resolving power, many compounds may still co-migrate and a single quadropole MS system can therefore fail to distinguish between two analytes having the same charge and molecular mass. The use of a triple quadropole MS system can overcome this problem by providing a second separation step (MS–MS). With this type of instrument, the second quadropole (Q2) serves as a collision cell where sufficient energy is transferred to the molecule to break chemical bonds producing fragment ions. By scanning the third quadropole (Q3) the masses of these fragments can be found and used to distinguish compounds with the same molecular mass.

An MS–MS instrument can thus find both the molecular mass and give structural information making accurate detection and identification easier. Where reference compounds are available, they can be infused directly into the instrument to find optimum MS–MS conditions and to identify the characteristic fragmentation pattern which can be used to create searchable reference libraries.

### *1.3. MS–MS modes of operation*

There are several MS–MS experiments that can be used to produce the analytical information of interest. The modes of operation used in this study, product ion scanning, precursor ion scanning, and multiple reaction monitoring (MRM), will be described here.

Fig. 1 (top) shows a schematic presentation of a product ion scan. This type of experiment is used to identify the fragmentation pattern of a compound and to optimize the Q2 collision energy (which is compound dependent). In this method, Q1 is set to let only ions of one specific mass/charge ($m/z$) ratio pass into the collision cell (Q2) where fragmentation occurs. The Q3 scans and acquires data from a low $m/z$ ratio to the $m/z$ ratio of the molecular ion (or higher if the precursor is multiply charged).

The principle of a precursor ion scan is shown in Fig. 1 (middle). This type of experiment is typically carried out to detect classes of compounds which yield a common fragment ion. This is accomplished by scanning Q1 to let all molecular ions pass to the collision cell while Q3 is set to let only the $m/z$ ratio of the common fragment ion pass to the detector. A spectrum with the characteristic $m/z$ ratios of all the compounds producing the specific fragment ion is produced.

The MRM mode follows the dissociation of a molecular ion to a specific fragment ion. Q1 is set to allow the molecular ion pass to the collision cell and Q3 is tuned to a characteristic fragment ion which is usually the most abundant ion. Sensitivity is greatly enhanced with MRM since the instrument focuses on a characteristic transition instead of scanning a large mass range.

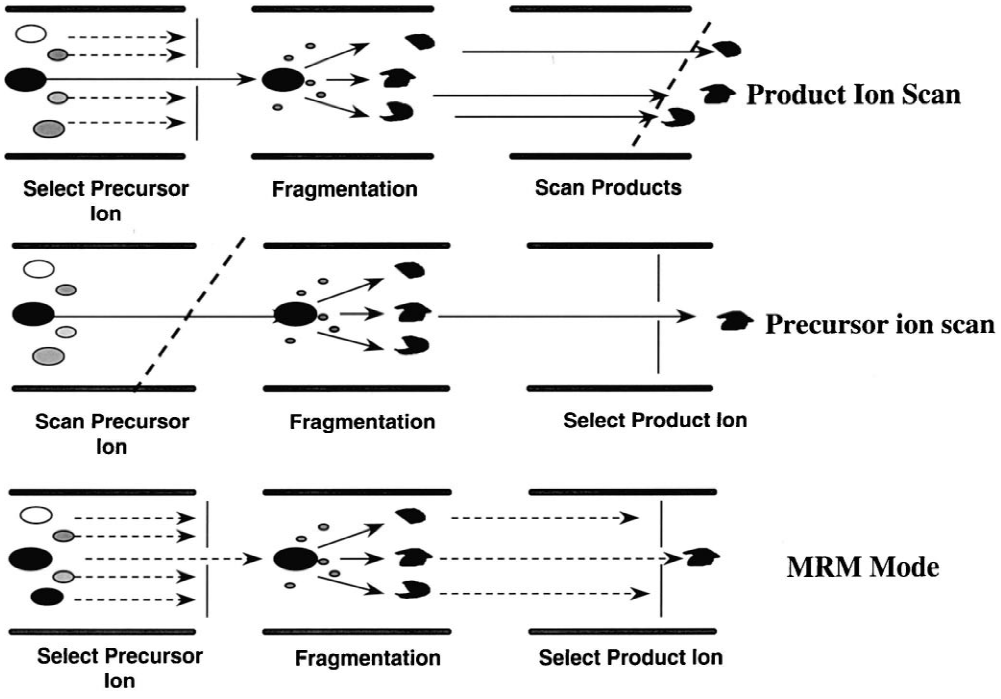


Fig. 1. MS–MS modes of operation.

### *1.4. The need for separation prior to MS–MS detection*

Fast and reliable MS–MS methods for screening amino acids and acylcarnitines from blood spots [17–20] have been developed during the last decade. This technique, often called neonatal screening, is currently used for screening for several inborn errors of metabolism by several hospitals world-wide. A chromatographic separation is not required before MS–MS analysis, and the sample is injected into the MS system as one plug through a high-performance liquid chromatography (HPLC) injector. In one run, both the amino acid profile (neutral loss of 102 u) and the acylcarnitine profile (precursor scan $m/z$ 85, $CH_2$–CH=CH–COOH cation) can be produced.

The neonatal screening methodology is well suited to detect approximately 20 of the more than 500 inborn errors of metabolism known today. Diseases like maple syrup urine disease, phenylketonuria, and medium chain acyl coenzyme A dehydrogenase deficiency (MCAD) can be diagnosed, but the total analysis time is quite long. Although the MS–MS analysis time is less than 2 min, the sample preparation takes more than 1 h.

As pointed out earlier, the detection of diagnostic metabolites in complex matrices, such as blood or urine, can be difficult due to the presence of native compounds of the same molecular mass. The neonatal screening method uses a long sample preparation step to eliminate interferences. Alternatively, an on-line sample separation prior to detection can be used. Although both CE and HPLC [21,22] are known to be suited for coupling to a mass analyzer, CE is recognized for having higher separation potential, shorter analysis time, and requires no sample preparation for the analysis of urines. With our experience using CE with DAD we therefore wanted to evaluate the potential of CE–MS–MS as a method for fast and accurate diagnosis of metabolic disorders.

## 2. Experimental

### *2.1. Urine samples*

Samples from patients with known metabolic disorders were analyzed and the results compared with control samples. The patient samples were taken from a bank of frozen urine samples from the clinical laboratory of our hospital. In this routine clinical laboratory, more than 1500 complete evaluations of patients, predominantly children, with symptoms indicating metabolic disorders are performed each year. Thus, for the patient samples studied in this work, the diagnoses were previously found using GC–MS, HPLC, an amino acid analyzer system, thin-layer chromatography or a combination of these techniques and confirmed by enzyme analysis and DNA mutation analysis. The creatinine concentrations of the urine samples were determined using a standard clinical chemistry method, and this value was transferred to the data acquisition system of the MS–MS for direct comparison of the results.

All samples were removed from the −20°C freezer and left at room temperature to thaw and mixed well before analysis. Samples with clearly visible precipitates were filtered through a 0.45-mm syringe filter prior to analysis.

### *2.2. Blood spots*

Blood spots from patients with propionic acidemia and very long-chain acyl coenzyme A dehydrogenase (VLCAD) deficiency were analyzed. Sample preparation was done according to the procedure described in Ref [20].; extraction of amino acids and acylcarnitines from filter paper by methanol, addition of deuterated internal standards, and derivatization with butanolic HCl to produce butylated amino acids and acylcarnitines that were injected directly into the MS–MS system.

### *2.3. CE*

CE separations were performed on a Crystal CE 300 (ATI Unicam) instrument. Samples were injected directly (or after filtering) at 100 mbar for 12 s. Non-coated, open fused-silica capillaries (Polymicro Technologies) with dimensions of 98 cm×50 μm I.D.×150 μm O.D. were used. Ammonium acetate, 20 m*M* at pH 8.5 was used as CE run buffer, and the applied voltage across the capillary was 25 kV, resulting in a current of approximately 20 μA.

Table 1
The masses, corresponding internal standards (I.S.s), and abbreviations for some selected carnitines analyzed by MS–MS

| Compound | Abbreviation | Mass | Mass I.S. |
|---|---|---|---|
| Free carnitine | $C_0$ | 218.3 | 227.3 |
| Acetylcarnitine | $C_2$ | 260.4 | 263.4 |
| Propionylcarnitine | $C_3$ | 274.5 | 277.4 |
| Isovalerylcarnitine | $C_5$ | 302.4 | 311.4 |
| Octanoylcarnitine | $C_8$ | 344.5 | 347.5 |
| Myristoylcarnitine | $C_{14}$ | 428.6 | 437.6 |
| Palmitoylcarnitine | $C_{16}$ | 456.6 | 459.6 |

### 2.4. MS

The CE system was interfaced with an API 2000 (PE Sciex, Canada) benchtop triple quadropole mass spectrometer, equipped with a patented LINAC high-pressure collision cell. Nitrogen was used as both the nebulizing and the collision gas. A coaxial sheath flow of 2 m*M* ammonium acetate was provided through the TurboIonSpray source at 5 μl/min in methanol–water (50:50).

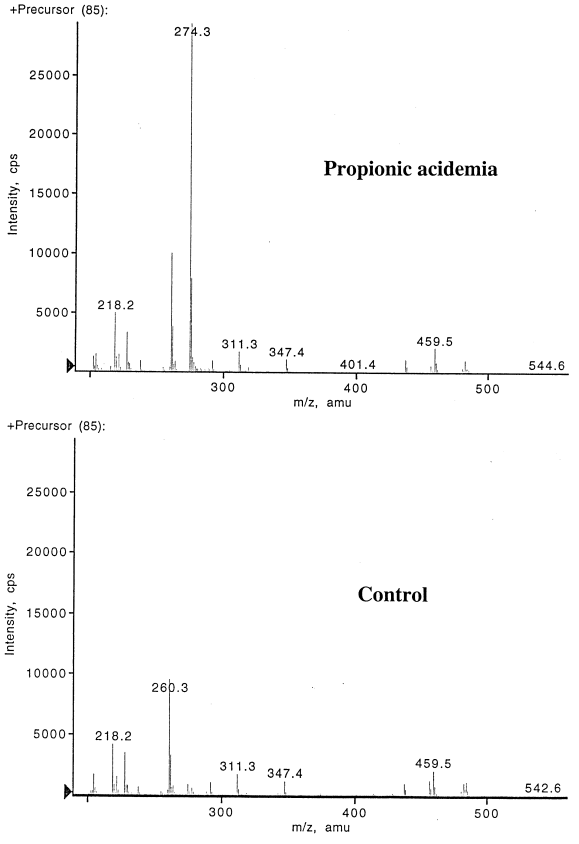


Fig. 2. MS–MS blood spot analysis of a propionic acidemia sample (top) vs. control (bottom). Experimental conditions: mass range: 200.2–550.6 by 1.0 u step size; dwell: 20.0 ms, 22 scans.

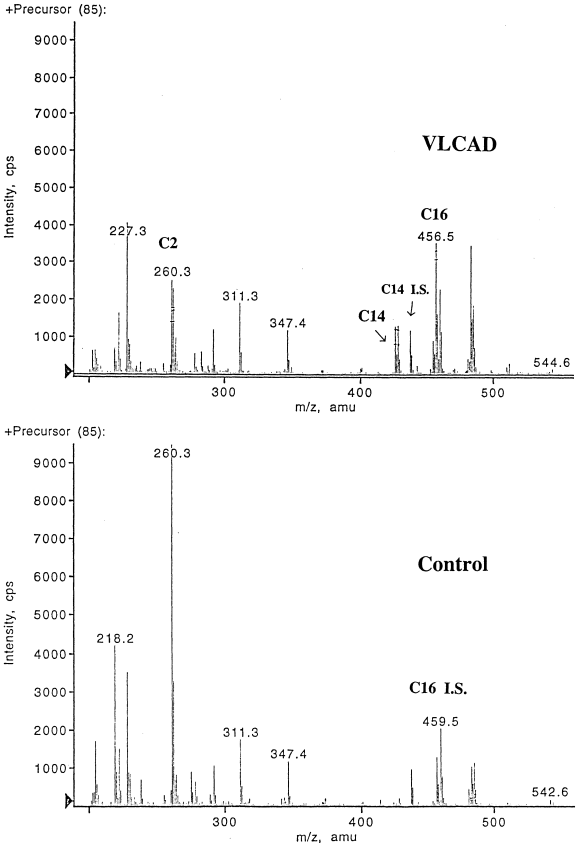


Fig. 3. MS–MS blood spot analysis of a VLCAD sample (top) vs. control (bottom). Experimental conditions as in Fig. 2. See Table 1 for identification of the different acylcarnitines.

## 3. Results and discussion

### 3.1. Examples on neonatal screening

Butyl esters of carnitines produce a common fragment of *m*/*z* 85 ($CH_2$–CH=CH–COOH cation) in MS–MS. By using a precursor ion scan of *m*/*z* 85, only the species giving rise to this fragment will be detected and thus yield the acylcarnitine profile of the blood spot sample. Table 1 lists some of the

acylcarnitines measured and their corresponding internal standards.

Fig. 2 (top) shows the MS–MS analysis of a blood spot from a patient suffering from propionic acidemia. The $C_3$ acylcarnitine ($m/z$ 274.3) accumulates in the blood of patients with this disorder, and as can be seen in the control (Fig. 2 bottom), this acylcarnitine is present at only very low concentration in healthy individuals. The analysis of a blood spot from a patient suffering from VLCAD is shown in Fig. 3 (top). The low concentrations of $C_2$ acylcarnitine ($m/z$ 260.3) and high concentration of $C_{14}$ and $C_{16}$ acylcarnitines ($m/z$ 428.6 and $m/z$ 456.5) were compared with the control sample (bottom) to confirm the diagnosis.

Table 2
Transitions found for some urinary metabolites using multiple reaction monitoring (MRM)[a]

| Compound | Transition |
|---|---|
| Propionic acid | 73→73 |
| Benzoic acid | 121→77 |
| Homogentisic acid | 167→123 |
| Homovanillic acid (HVA) | 181→137 |
| Vanillyl mandelic acid (VMA) | 197→137 |
| Glyceric acid | 105→75 |
| Orotic acid | 155→111 |
| Creatinine | 112→41 |
| Hippuric acid | 178→134 |
| *p*-Hydroxyphenyllactic acid | 181→163 |
| Galactose | 179→179 |

[a] Collision energy: 15 kV.

### *3.2. MRM of urinary metabolites*

Product ion scan experiments of the target compounds were performed to determine the specific transitions characteristic of each compound (Fig. 4). The selected transitions were used to perform MRM experiments. Table 2 lists the resulting transitions for some of the urinary metabolites studied.

Fig. 5 (top) shows the CE–MS–MS total ion electropherogram of a mixture of six reference compounds. Although they are not well separated in time, by running CE–MS–MS in the MRM mode,

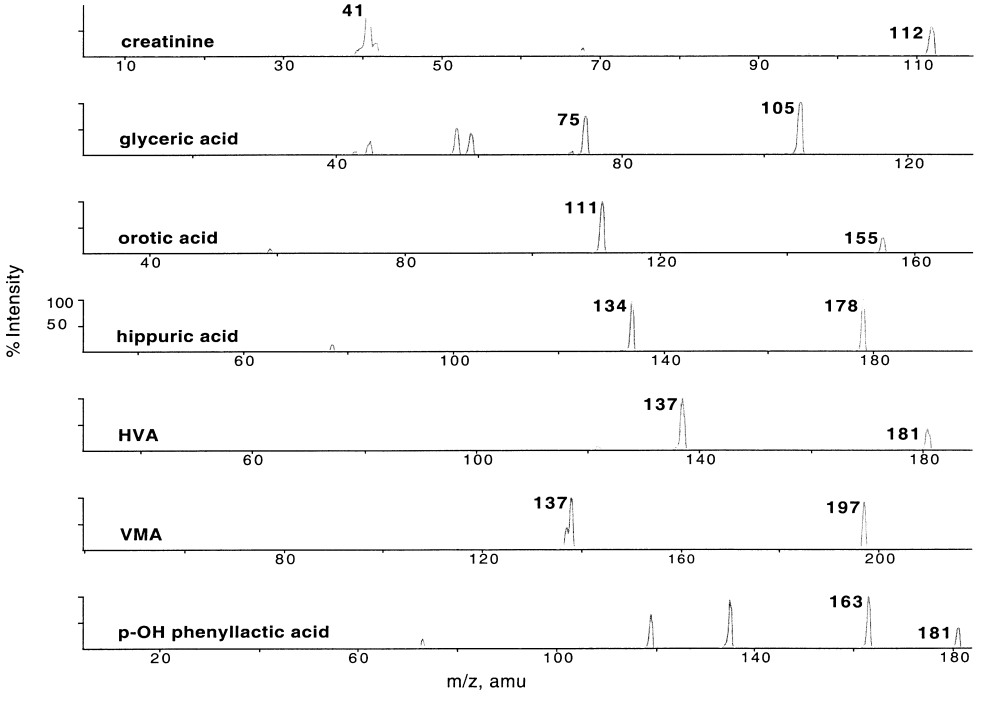


Fig. 4. Product ion scan of reference compounds of the urinary metabolites creatinine, glyceric acid, orotic acid, hippuric acid, homovanillic acid (HVA), vanillylmandelic acid (VMA), and *p*-hydroxyphenyllactic acid.

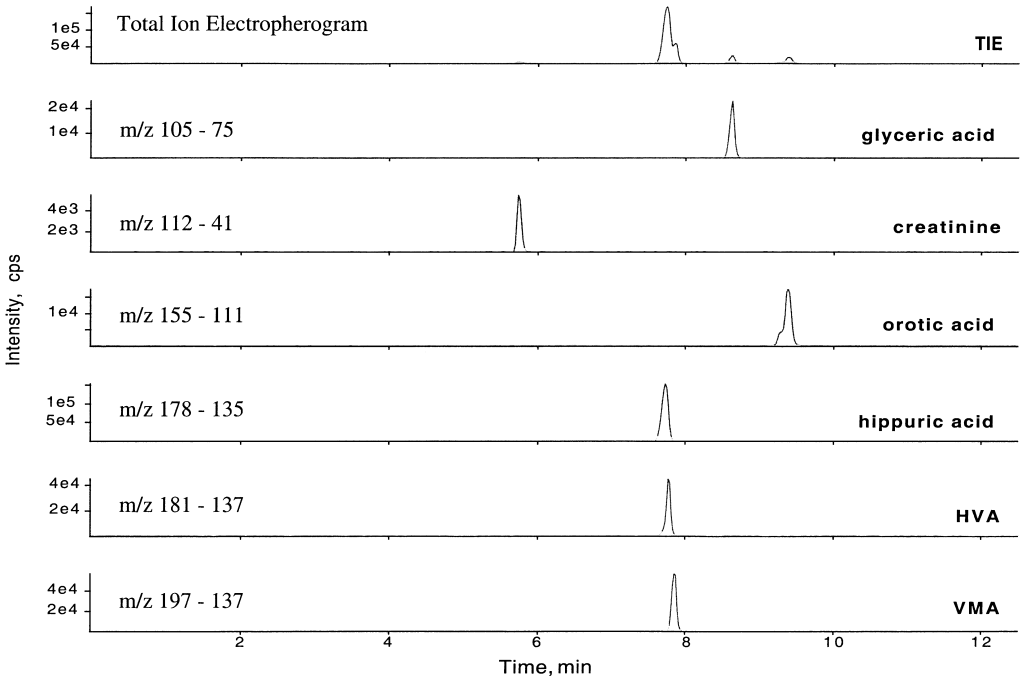


Fig. 5. CE–MS–MS of six reference compounds of urinary metabolites.

the different compounds can be easily separated and identified based on their characteristic MS–MS transitions (Fig. 5, bottom).

### *3.3. CE–MS–MS (MRM) analysis of patient samples*

By using CE–MS–MS in the MRM mode, the diagnostic metabolites of several different disorders were identified in urine samples (examples are listed in Table 3). Fig. 6 shows the results of the CE–MS–MS analysis of a galactosemia patient urine compared with control urine. The metabolic disorder galactosemia results in increased urinary excretion of galactose, galactitol and galactose-1-phosphate [23]. Untreated patients will rapidly develop liver failure, which results in the excretion of a secondary metabolite, *p*-hydroxyphenyllactic acid. This secondary metabolite shows high UV absorption, and we have previously shown [9] that it can easily be detected using the simple CE–DAD method. The primary diagnostic metabolite, galactose, does not absorb UV light and is therefore not identified using this method. The results in Fig. 6 show that both the primary (galactose) and the secondary metabolite (*p*-hydroxyphenyllactic acid) were present in the patient urine at high concentrations, but nearly absent from the control urine (reference interval for galactose: $<0.08$ mmol/day [24]). This patient urine was therefore a good candidate as a test of the usefulness of CE with MS–MS detection.

Table 3
Some diagnostic metabolites and their corresponding diseases found using CE–MS–MS

| Diagnostic metabolite | Disease |
|---|---|
| Benzoic acid | Urinary tract infection |
| Homogentisic acid | Alcaptonuria |
| Homovanillic acid (HVA) | Neuroblastoma |
| Vanillyl mandelic acid (VMA) | Neuroblastoma |
| Galactose | Galactosemia |
| *p*-Hydroxyphenyllactic acid | Galactosemia |
| $C_{12}$ and $C_{14}$ epoxy acids | Zellweger syndrome |
| Propionic acid | Propionic aciduria |
| Methyl malonic acid | Methyl malonic aciduria |
| Orotic acid | HHH syndrome |

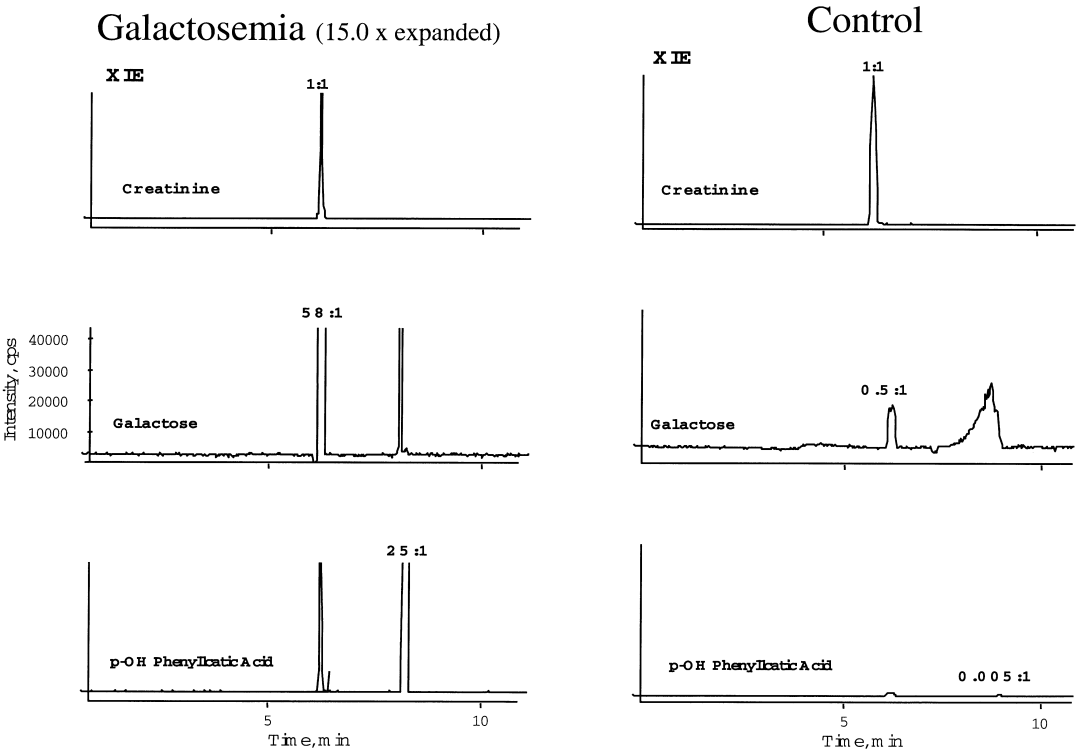


Fig. 6. CE–MS–MS (MRM mode) of a galactosemia sample (left) vs. control (right). The creatinine value of the control sample was 15-times higher than the patient sample, and to make direct comparison possible the galactosemia results had to be expanded 15 times. The ratio of the different metabolites are shown.

Homovanillic acid (HVA) and vanillylmandelic acid (VMA) [reference intervals [24] (3–6 years): HVA: 3.4–9.6 mmol/mol creatinine, VMA: 5–13 μmol/day] are diagnostic metabolites for the disease neuroblastoma [25]. Using the same methodology, HVA and VMA were identified in the urine from a neuroblastoma patient (Fig. 7). Again, these metabolites were barely detected in the control sample.

Patients with Zellweger syndrome excrete $C_8$–$C_{14}$ epoxy acids in their urine [26] whereas the serum has elevated levels of very long-chain fatty acids. The CE–MS analysis of a patients' urine made it possible to detect the presence of the $C_{12}$ and $C_{14}$ epoxy acids (Figs. 8 and 9) by monitoring their calculated negatively charged molecular ions without the use of reference compounds. These peaks were not detected in the control urine (not shown). To obtain stronger evidence that these were the actual epoxy acids, product ion scans of the peaks were acquired. Similar fragmentation pattern for the two peaks were expected (Fig. 9) since the chemical structure of the epoxy acids differ only in chain length. This is a strong indication that the peaks detected are the diagnostic metabolites characteristic of the peroxisomal disorder Zellweger syndrome.

## 4. Concluding remarks

Our results suggest that both CE–DAD, MS–MS and CE–MS–MS have high potential as powerful tools in laboratories aimed at the diagnosis of metabolic disorders. The main goal is to find faster

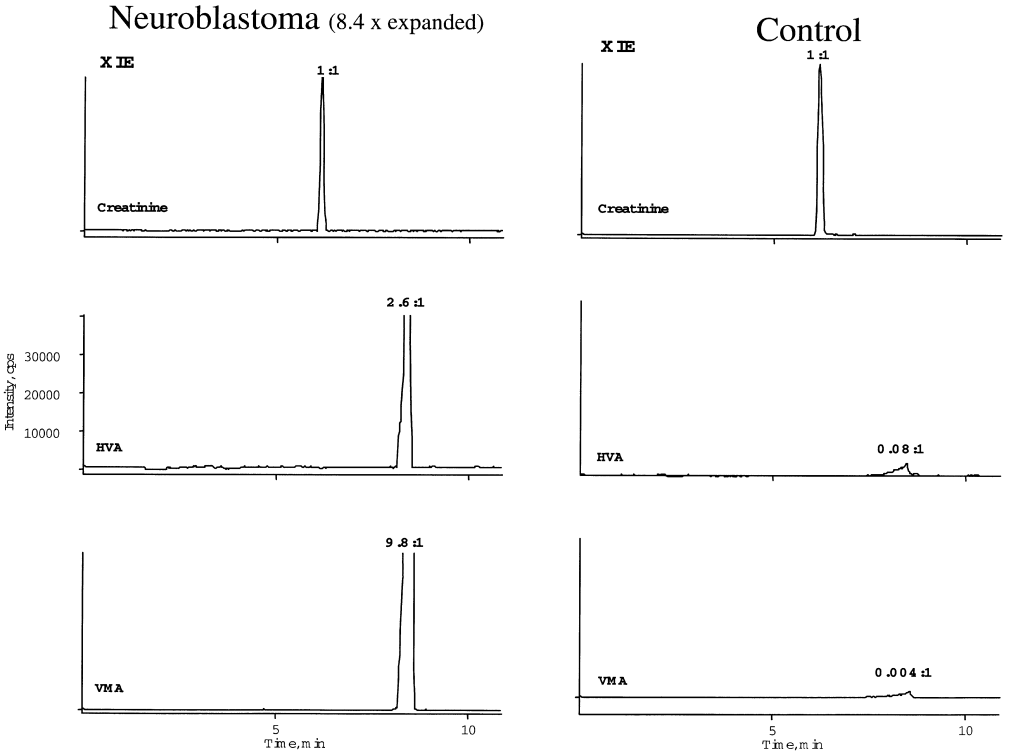


Fig. 7. CE–MS–MS (MRM mode) of a neuroblastoma sample (left) vs. control (right). See Fig. 6 for description of layout.

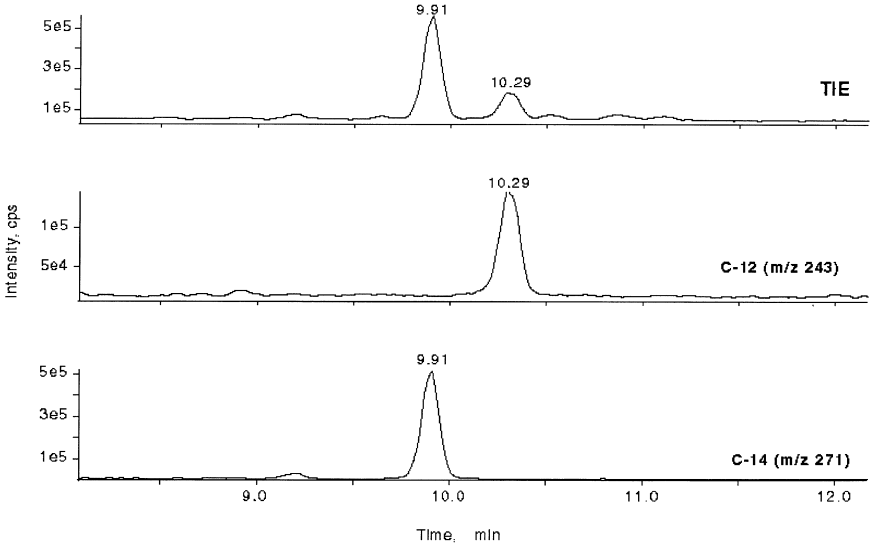


Fig. 8. CE–MS–MS total ion electropherogram (top) and selected ion monitoring of $m/z$ 243 and $m/z$ 271, the calculated negatively charged ion of $C_{12}$ and $C_{14}$ epoxy acid in the Zellweger syndrome sample.

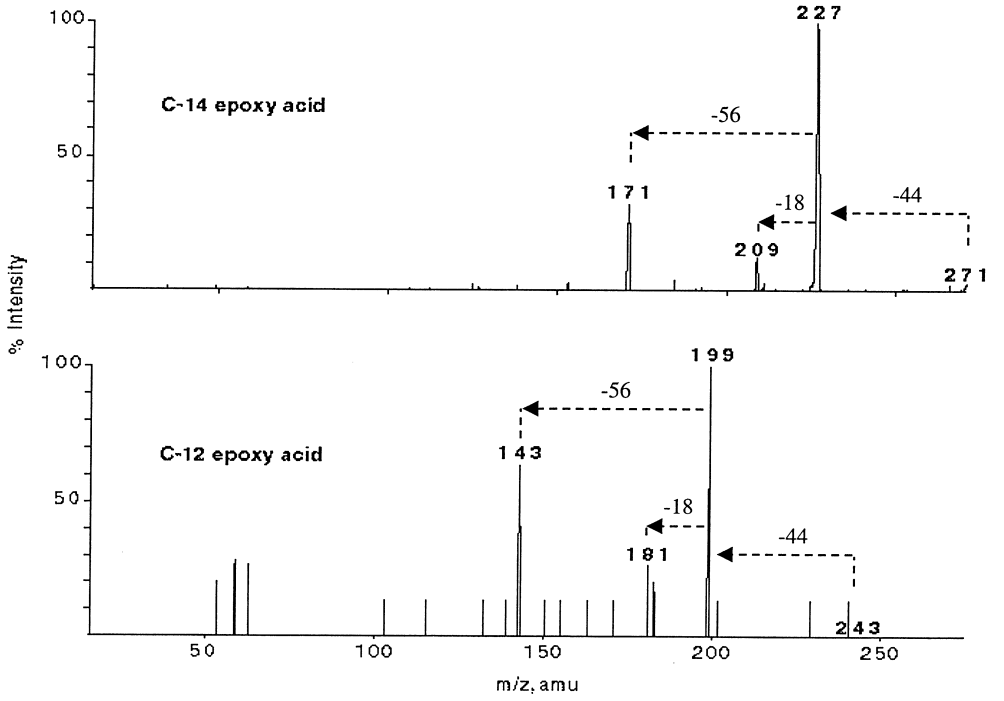


Fig. 9. MS–MS fragmentation pattern of the $m/z$ 243 and $m/z$ 271 peaks shown in Fig. 8.

and more reliable methods for the identification of diagnostic metabolites in patients with symptoms indicating metabolic disorders, and to diagnose the large number of disorders that current routine methods are not capable of detecting.

The background clinical history and preliminary investigations including standard clinical chemical analyses will help determine which of the above techniques should be used. Where the expected diagnostic metabolite has an electrophoretic migration that differs sufficiently from most other urinary metabolites, patient samples can be run on CE–DAD, and if necessary the identity of the diagnostic metabolite can be confirmed with direct infusion MS–MS. In other cases where the diagnostic metabolites are not well separated using CE alone, or when the diagnostic metabolites do not absorb UV light, CE–MS–MS will be the method of choice. The MS–MS neonatal screening approach will most likely be the method of choice when the symptoms and results of preliminary clinical tests indicate defects that can be recognized from the acylcarnitine or amino acid profile.

Although the results shown are promising, the potential of these techniques must be further evaluated by running patient samples routinely and in parallel with existing techniques (where available) over a length of time to fully reveal their merits and limitations. In addition, CE–ESI-MS–MS libraries of urinary metabolites must be created for automated identification of metabolites when running unknown samples.

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