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Application of capillary electrophoresis and related techniques to drug metabolism studies

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Abstract

The use of capillary electrophoresis (CE) for the separation of small organic molecules such as pharmaceutical agents and drug/xenobiotic metabolites has become increasingly popular. This has arisen, at least in part, from the complimentary mode of separation afforded by CE when compared to the more mature technique of HPLC. Other qualities of CE include relative ease of method of development, rapid analysis, and low solvent consumption. The recent introduction of a variety of detector systems (including UV diode array, laser-induced fluorescence, conductivity) and the demonstrated coupling of CE to MS have also aided acceptance of this technology. In the present report, we review the role of CE coupled to various detector systems including a mass spectrometer for the characterization of both in vitro and in vivo derived drug metabolite mixtures. Attributes of CE for this application are demonstrated by discussion of metabolism studies of the neuroleptic agent haloperidol. Various aspects of the development and use of CE and CE–MS for the characterization of haloperidol metabolites, including criteria for selection of parameters such as pH, ionic strength, extent of organic modification, and the use of nonaqueous capillary zone electrophoresis are discussed. We also consider potential limitations of CE and CE–MS for drug metabolism research and describe the introduction of membrane preconcentration–CE (mPC–CE) and mPC–CE–MS as a solution that overcomes the rather poor concentration limits of detection of CE methods without compromising the resolution of analytes or separation efficiency of this technique.

Keywords: Drug metabolism; Membranes; Sample preparation; Detection, electrophoresis; Pharmaceutical analysis; Drugs; Haloperidol

1. Introduction

The structural diversity of the multitude of modern therapeutic drugs available for the treatment of disease is well documented [1,2]. However, after entry into the body, such therapeutic agents are subjected to metabolism. This latter process involves a series of complex events, including absorption, distribution, metabolic transformation and, eventually, excretion of the remaining parent drug and/or its metabolites. These events modulate the efficacy of the administered drug in the treatment of the disease, as well as determining its pharmacological and toxicological effects. Therefore, the subsequent isolation and structural characterization of the resulting plethora of metabolites is vitally important to understanding the physical and biological effects of the parent drug [3–5].

The enzymatic reactions involved in drug metabolism pathways are often classified into three groups

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Table 1
Classification of phase I, II, and III drug metabolism

Phase I	Phase II	Phase III
Oxidation	*Glucuronidation* at –OH, –COOH, $-NH_2$, –SH	e.g., Glutathione-S conjugates of haloalkanes
Aliphatic oxidation		
Aromatic hydroxylation		
N–, O–, S–dealkylation	*Acetylation* at $-NH_2$, $-SO_2NH_2$, –OH	$\xrightarrow{\beta\text{-lyase}}$ Unstable thiols
Epoxidation		
N–, S–oxidation	*Glycosylation* at –OH, –COOH, –SH	
Dehalogenation		
Alcohol and aldehyde oxidation	*Methylation* at $-NH_2$, –OH, –SH	
Oxidative deamination		
	Sulfation at $-NH_2$, $-SO_2NH_2$, –OH	
Reduction	*Glutathione conjugation*	
Azo-, nitro reduction		
	Fatty acid conjugation	
Hydrolysis		
Amide, ester, hydrazide and carbamate hydrolysis		
Epoxide hydration		
Isomerization		

(see Table 1). Phase I metabolism consists of a variety of functionalization reactions, including oxidation, reduction, and hydrolysis, which generally produces compounds of enhanced polarity that are subsequently susceptible to further modification. Phase II metabolism involves conjugation of the drug, or more likely a phase I metabolite, with an endogenous substrate, such as the tripeptide glutathione or glucuronic acid. This normally leads to a water-soluble product which can then be more readily excreted in bile or urine. The existence of a third phase of metabolism involving breakdown of conjugates by intestinal microflora accompanied by subsequent reabsorption and metabolism (see Table 1) has also been postulated [6].

Contemporary methods for drug metabolite identification are usually based on the comparison of ultraviolet (UV) spectral data and high-performance liquid chromatography (HPLC) retention times of isolated "unknown" metabolites with synthetic standards [7]. Such methods of detecting drug metabolites and subsequent structural characterization can be a time-consuming process, as well as only affording very limited structural information. Furthermore, since phase I metabolism of a drug often results in only minor structural modification of the parent compound [3,4], determination of suitable chromatographic conditions to effect HPLC separation of mixtures of drug metabolites is often particularly difficult. Also, since phase II conjugate metabolites are generally thermally unstable and very polar, they are difficult to assay by classical methods, such as HPLC. To this end, the high-efficiency separations of capillary electrophoresis (CE) combined with the ease of rapid analysis and method development presents obvious advantages over the use of HPLC for the detection of new and possibly relatively short-lived reactive drug metabolites. CE also offers the unique possibility for simultaneous analysis of both phase I and phase II metabolites.

The use of CE for the analysis of small organic molecules (M_r<ca. 1000) such as conventional pharmaceutical agents and drug metabolites has started to receive considerable attention. A number of recent reviews have been published summarizing the role of CE in the analysis of such compounds [8–17]. Furthermore, a wide variety of such pharmaceutical agents, drugs and physiologically-derived metabolites have been analyzed by either free solution capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MECC), as summarized in Table 2.

A variety of CE detection devices other than UV have been utilized in the analysis of small molecules.

Table 2
Types of pharmaceutical agents, therapeutic drugs and physiologically derived metabolites analyzed by free solution CZE or MECC

Compound type	Method (Source)[a]	Reference
Amphetamines	CZE–MS[b] (urine)	[35]
Anesthetics	MECC (serum)	[69]
	MECC (serum)	[70]
	CZE (brain microdialysate)	[71]
Anthelmintics	CZE (serum)	[72]
Antibiotics	CZE	[24]
Cephalosporins	CZE	[73]
	MECC	[74,75]
	MECC (plasma)	[76]
	CZE (urine)	[77]
	MECC	[78]
Penicillins	CZE	[73]
	MECC	[74,75]
	MECC (plasma)	[74]
	MECC (plasma)	[79]
Sulfonamides	CZE	[73]
	CZE–MS, CZE–MS–MS	[34]
	CZE (pork meat)	[80]
	CZE	[81]
Tetracyclines	CZE	[82]
	MECC	[83]
Anticancer	CZE	[84]
	CZE–UF[c] (serum)	[18]
	CZE	[85]
	CZE, MECC (various)	[86]
Anticonvulsants	CZE (serum)	[87]
Antidepressants	CZE	[53]
	MECC (plasma)	[88]
Antiepileptic	MECC (serum)	[89–91]
	MECC (serum, saliva, urine)	[92]
Anti-inflammatories	CZE	[73,93]
	MECC	[74,93,94]
	MECC (cyclodextrin)	[95]
	CZE, MECC	[96]
	CZE/chiral	[97]
	CZE	[98]
Antileukemic	Chiral EEC	[99]
	CZE (serum)	[100]
Antimalarial	CZE, MECC	[101]
Antipyrine	CZE (saliva)	[102]
Anxiolytic	CZE	[103]
Barbiturates	MECC	[70,73]
	MECC (plasma, urine)	[25]
	MECC (plasma, serum)	[70]
	CZE (serum)	[104]
	CZE (serum)	[105]
Benzodiazepines	CZE–MS, CZE–MS–MS	[34]
	MECC (urine)	[106]

(Continued on p. 418)

Table 2 (*continued*)

Compound type	Method (Source)[a]	Reference
Beta-blockers	CZE (serum)	[107]
	CZE (urine)	[108]
	MECC (urine)	[109]
Calcium channel blockers	MECC	[74,94]
Cardiovascular	MECC	[110]
Cold medicines and analgesics	CZE	[73,111]
	MECC	[74,111–113]
	CZE (serum, urine)	[114]
	MECC (serum)	[115]
	MECC (urine)	[116]
Cytokines	CZE	[117]
Diuretics	CZE	[118]
	CZE (serum)	[119]
H_1-Antagonists	CZE	[120]
H_2-Antagonists	MECC (serum)	[121]
	CZE (plasma)	[122]
	CZE–MS (microsomal incubation)	[43,44,123]
Mucolytic agents	CZE and ITP[d]	[124]
Neurological agents	CZE, CZE–MS, CZE–MS–MS (microsomal incubation)	[39–41,49]
	CZE–MS (urine)	[41]
	CZE (plasma)	[125]
Physiological metabolites		
Methylmalonic acid	CZE–LIF (serum)	[19]
Porphyrins	MECC (urine)	[126]
Urea and creatinine	CZE (urine)	[127]
	CZE (serum)	[128]
Solid-tumor agents	CZE, CZE–MS (urine)	[45,46]
Vitamins		
Vitamin A	CZE–LIF (serum)	[20]
Vitamin B	CZE	[111]
	MECC	[74]
	MECC (blood)	[21]
	MECC (urine)	[22]
	CZE, MECC	[129]
	CZE	[130]
Vitamin C	CZE (urine, plasma)	[131]
Uric acid	CZE	[132]
	CE–MS[b] (urine)	[35]
	CZE (urine, serum)	[133]
	MECC (urine)	[134]
Xanthines	MECC	[132]
Illicit drugs	MECC (urine)	[135,136]
	MECC (serum, saliva, urine)	[26,137]
	MECC (hair)	[92]
	CZE, MECC (hair)	[138,139]

[a] Unless otherwise stated, commercial source.
[b] CE–MS, off-line analysis by MS.
[c] LIF, laser induced fluorescence detection.
[d] ITP, isotachophoresis analysis.

These include laser-induced fluorescence (LIF) [18–22], electrochemical [23] and multi-wavelength UV array detectors [24–27]. The limitation of these detectors is the lack of structural information obtained, particularly when compared to mass spectrometry (MS). Considering all the currently available detection systems capable of analyzing sub-nanogram quantities, MS is the most powerful technique presently used for acquiring structural data on unknown compounds. Furthermore, the advent of ion array detectors coupled to mass spectrometers has significantly enhanced the capabilities of such instruments with attomole detection levels now attainable [28]. However, the use of on-line CE–MS and CE–tandem mass spectrometry (CE–MS–MS) for the analysis of such molecules has not been fully exploited.

The development of on-line CE–MS, pioneered independently by Smith and co-workers [29,30] and Henion and co-workers [31–33], has further enhanced the utility of CE in the analysis of complex drug metabolite mixtures. However, to date, there have been only a small number of studies reporting the use of CE–MS in drug and metabolism studies. Johannson et al. investigated the separation and mass spectral analysis of a series of sulfonamide and benzodiazepine metabolites, as well as the in vivo metabolic fate of flurazepam [34]. Guzman et al. used two different preconcentration steps and analyte fractionation on-line with CE to analyze uric acid and methamphetamine in human urine by off-line electron ionization MS [35]. Perkins et al. and Parker et al. have reported the separation of a series of sulfonamide drugs [36] and macrolide antibiotics [37], respectively. More recently, Sheppard et al. have reported the separation and characterization of chiral drugs by CE–MS [38]. We have published a series of CE–MS and CE–MS–MS studies on a number of therapeutic drugs including the neuroleptic agent haloperidol (HAL) [39–41] the H_2-antago-

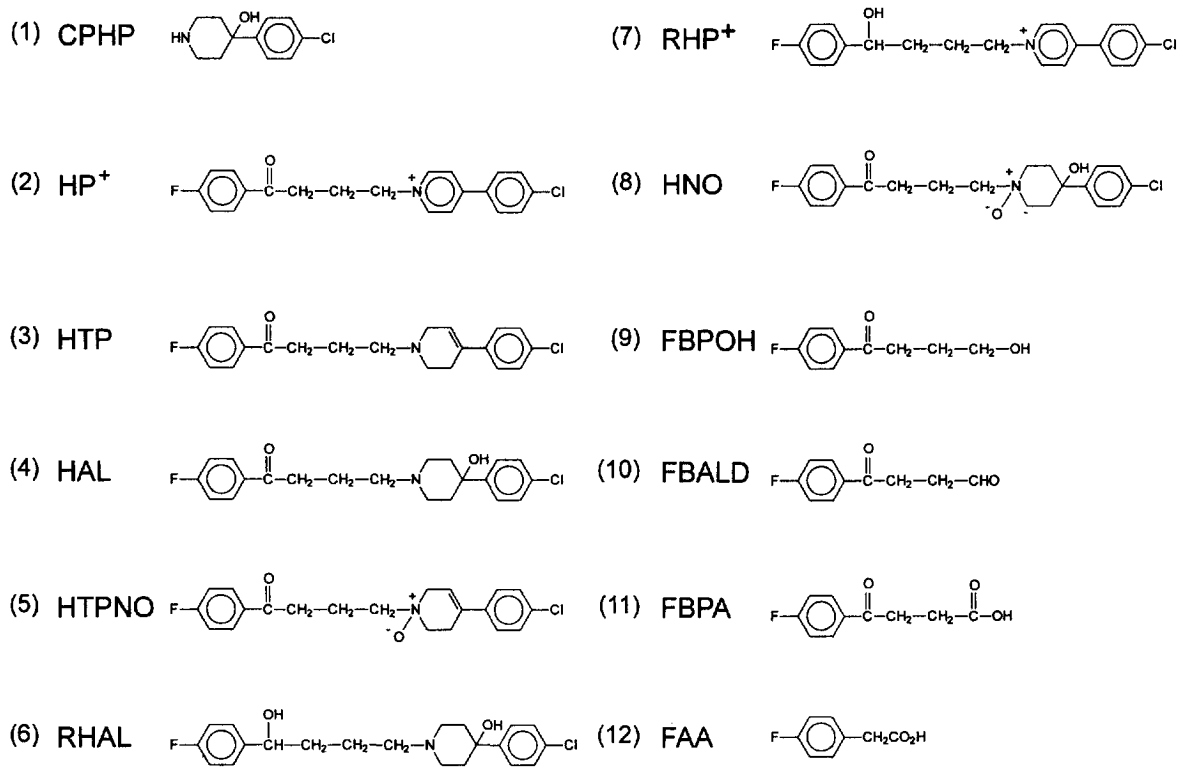

Fig. 1. Structures of the neuroleptic drug HAL and eleven synthetic analogs/putative metabolites. Note that the compounds are numbered and the assigned number is used consistently throughout the figures.

nist mifentidine [42–44] and the anti-tumor compound pyrazoloacridine [45,46].

In the present work, we present a systematic approach to structurally characterize biologically derived metabolites of the neuroleptic drug HAL (see Fig. 1 for structure). In particular we describe the development of capillary zone electrophoresis (CZE), CZE–electrospray-MS, (CZE–ESI-MS), and CZE–ESI-MS–MS conditions to effect optimal separation and, ultimately, structural determination of in vivo derived metabolites of HAL in human urine samples. Adopting this strategy, we highlight some of the potential limitations associated with the analyses of drug metabolite mixtures by CE and CE–MS, and describe some of our approaches to overcome such problems.

2. Experimental

2.1. Materials, chemicals and synthetic standards

HAL (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol), potassium phosphate (monobasic), zinc sulphate, ammonium hydroxide, and potassium hydroxide were obtained from Sigma (St. Louis, MO, USA). Gold-grade ammonium acetate, glacial acetic acid (99.9% grade), and magnesium chloride were obtained from Aldrich (Milwaukee, WI, USA). NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were supplied by Boehringer Mannheim (Indianapolis, IN, USA). HPLC-grade solvents methanol, acetonitrile, water, and methylene chloride were obtained from Baxter (Minneapolis, MN, USA). High-purity water was purchased from Burdick and Jackson (Muskegon, MI, USA). 4-(4-Chlorophenyl)-4-hydroxypiperidine (CPHP), 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxybutyl]-4-piperidinol N-oxide (HNO), 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridine (HTP), 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridine N-oxide (HTPNO), 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium (HP^+), 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-4-piperidinol (RHAL), 4-fluorobenzoyl propionic acid (FBPA), 4-fluorobenzoyl propanol (FBPOH), 4-fluorobenzoyl propanal (FBALD) and 4-fluorophenyl acetic acid (FAA), were all synthesized as described previously [47]. 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-1-pyridinium $(RHP)^+$ was a gift from Prof. N. Castagnoli Jr. (VPIST). The 3M Empore polymeric styrene–divinylbenzene copolymer (PS–DVB) membranes were obtained from Varian (Harbor City, CA, USA). Polyimide-coated fused-silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ, USA). PTFE tubing was obtained from Chromtech (Apple Valley, MN, USA).

2.2. CZE

2.2.1. Single-wavelength UV detection

CZE separations were performed on a modified Beckman P/ACE 2100 CE instrument (Fullerton, CA, USA) coupled to a Reason Technology 486 PC (Rochester, MN, USA) with system control and data capture by System Gold software (Beckman). Uncoated capillaries (various lengths and I.D.), purchased from Beckman were used throughout the analysis. Prior to use, each capillary was rinsed with 10 capillary volumes of 1 *M* NaOH, 1 *M* HCl, water, MeOH and finally separation buffer. Between analyses, capillaries were rinsed with MeOH (2 capillary volumes) and subsequently separation buffer (2 capillary volumes). Synthetic standards were individually dissolved in MeOH (~1 μg/μl) and 5 μl was removed from each vial and mixed in a single, clean vial to give a synthetic mixture containing parent drug and standards. Samples were introduced into the CE capillary by pressure injection (1–10 s, see individual figure legends) and all experiments were conducted using an applied voltage of 20, 25, or 30 kV and a capillary temperature of 25°C. Analytes were monitored at a wavelength of either 200 or 214 nm. Separation buffers used are described in individual figure legends.

2.2.2. Diode array detection (DAD)

CE separations were performed on a modified Beckman P/ACE 5000 model with a diode array detector. The separation runs were monitored at 214 nm, and a data acquisition rate of 2 Hz. Data points were collected every 2 nm from 200 to 300 nm. Data

was analyzed using the scan graphics software obtained from Beckman. All other conditions were the same as for CE analysis using the single-wavelength UV detector, except that the applied voltage was reduced to 20 kV.

2.3. CZE–ESI-MS and CZE–ESI-collision induced dissociation-MS (CZE–ESI-CID-MS)

All analyses were carried out on a Finnigan MAT 900 mass spectrometer (Bremen, Germany) of EB configuration (where E is an electrostatic analyzer and B is the magnet) equipped with a PATRIC (position and time resolved ion counter) scanning array focal plane detector. Either a Finnigan MAT ESI source or modified Analytica (Banford, CT, USA) source was used. The former device employs a spray needle that is floated to voltage (typically 6–8 kV) and a heated metal capillary (~200°C) as the first stage of separation of the atmospheric (API) spray region and the vacuum of the mass spectrometer. A skimmer is positioned beyond this capillary as a second stage of separation between the API region and the MS vacuum. Ions that transfer into the MS ion source initially enter an octapole that aids focusing. In the latter source, the needle assembly is held at ground potential.

The modified source was used in a positive ion mode throughout, and the sample needle of the ESI source was replaced by the CE capillary from which 2–3 mm of the polyimide coating had been removed from the MS end with hydrofluoric acid. A liquid sheath electrode of isopropanol–water–acetic acid (60:40:1, v/v/v) at a flow-rate of 3 μl/min was used to boost the flow through the ESI needle and serve as a ground for the CE capillary. The nitrogen ESI drying gas was at 140°C and a flow-rate of 3.6 l/min and an ESI voltage of −3400 V was employed. For CE–MS analyses on in vitro derived samples, the scan range was 125–450 daltons (exponential magnet scan from low to high mass) at a rate of 3 s/decade, while CZE–ESI-CID-MS experiments were performed with a scan range of 60–450 daltons at a rate of 2 s/decade. The capillary and tube lens voltages were typically ~140 V and 210 V, respectively. An instrument resolution of ~1200 was employed throughout.

2.4. Membrane preconcentration–CE–MS (mPC–CE–MS)

The preconcentration capillary used in these experiments was prepared from uncoated fused-silica tubing, pretreated with potassium methoxide, methanol and finally CE separation buffer. A piece of polymeric (styrene divinylbenzene, SDB) membrane was installed at the mid-point of a PTFE cartridge. A fused-silica capillary was set into each end of the cartridge with solvent-resistant epoxy resin. Prior to installation, the membrane was activated by washing with MeOH, then CE separation buffer. The entire mPC–CE capillary was then conditioned under high pressure ($138 \cdot 10^3$ Pa, 20 p.s.i.) for 10 min with CE separation buffer. All subsequent capillary treatments and sample loading, washing, and elution were also carried out under high pressure ($138 \cdot 10^3$ Pa, 20 p.s.i.).

The method of analysis included a cleaning regime of methanol (0.2 min) and separation buffer (5 min), followed by a high-pressure injection (0.1–2 min) of the mixture to be analyzed. The capillary was then washed with separation buffer for 5 min and analytes were eluted from the packing material with either MeOH–CH_3CN (1:1) or 100% methanol followed by a plug of CE separation buffer. CE separations were performed using a Beckman P/ACE 2100 coupled via a Beckman CE–MS power supply and interfaced to a MAT 900 mass spectrometer.

2.5. CZE–fast linked scanning-MS–MS (CZE–FLS-MS–MS) and mPC–CZE–FLS-MS–MS

After analytes were either injected onto the CE capillary or eluted from the mPC–CE cartridge, they were subsequently subjected to CZE separation (as described above) and linked scan analyses were then conducted on the MAT 900. The protonated molecule (MH^+) was used as the link mass and attenuated to 40% of its original intensity using helium as the collision gas. Product ions were measured over the mass range 50–410 daltons at 2 s/decade using the linked scan function B/E=constant. Ions were detected using the PATRIC detector in scanning mode with either a 2 or 8% mass window.

2.6. Microsomal incubations

English short-hair male guinea pigs were obtained from Charles River (Montreal, Canada). Animals were fasted overnight before sacrifice. Hepatic microsomal preparations were prepared using the centrifugation method described previously [47]. Incubation procedures were as follows: a nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisting of the sodium salt of NADPH ($NADP^+$) (2 μmol), glucose-6-phosphate disodium salt (20 μmol), glucose-6-phosphate dehydrogenase (1 unit), and $MgCl_2$ (2 mg) all in 2 ml phosphate buffer (0.2 *M*, pH 7.4) was preincubated for 2 min. Enzymatic reactions were initiated by addition of HAL (2 μmol) and microsomal preparations equivalent to 0.5 g original tissue. In control incubations, heat-inactivated microsomes were used instead of fresh microsomal preparations. Incubations were carried out for 30 min at 37°C.

Enzymic reactions were terminated by addition of $ZnSO_4$ (200 mg) to the incubation mixture. The precipitated proteins were removed by centrifuging (IEC Cru-5000) at ~1200 *g* for 20 min. The supernatant was passed through a preconditioned [methanol (4 ml) followed by distilled water (4 ml)] Sep-Pak C_{18} cartridge. Excess $ZnSO_4$ was removed by washing with distilled water (4 ml). The retained compounds were eluted by methanol (4 ml), which was subsequently evaporated to dryness at 45°C under N_2 [48]. The residues were reconstituted in methanol (200 μl) and subjected to CZE–MS.

2.7. Urine sample analysis

Urine was obtained from a 27 year-old female patient receiving 0.5 mg of HAL per day. Specimen samples were frozen at −20°C until prepared for analysis. Urine (3 ml) was passed through a preconditioned [methanol (4 ml), followed by distilled water (4 ml)] C_{18} Sep-Pak cartridge. The Sep-Pak was washed with distilled water (4 ml) and retained analytes eluted with methanol (4 ml). The methanol fraction was dried at 45°C under N_2 and stored at −20°C until analysis by CZE–MS, when it was redissolved in 10 μl methanol–water (1:1).

3. Results and discussion

The structural diversity of the multitude of modern drugs available for the treatment of disease is well documented [1]. Hence the evolution of methods such as HPLC, for the separation of drug metabolite mixtures derived from both in vivo and in vitro sources has tended to be specific for a particular drug or class of drugs. In a previous study, we reported CZE separation buffer conditions that could be employed to resolve the metabolites of a wide variety of drug types based upon consideration of simple structural features of the parent drug and the resulting metabolites [49]. Also, these separation buffer conditions were developed in order to be compatible with ESI-MS so as to enable on-line CE–ESI-MS. Therefore, the use of MECC separation conditions was precluded since surfactants, such as sodium dodecyl sulphate (SDS) which are commonly used in this technique are not compatible with ESI-MS as they lead to suppression of analyte ionization. Hence, only CZE volatile separation buffers were investigated and two simple factors were considered to effect the separation of parent drug and metabolites, namely the hydrophobic character of the drug and the ionizable functional groups of analyte molecules.

3.1. Determining initial CZE conditions

The hydrophobicity of a compound is frequently expressed as its partition ratio between octanol and water. However, a qualitative determination of the hydrophobic character of molecules is readily assignable from consideration of functional groups present. Aromaticity and aliphatic character clearly determine the hydrophobic nature of the molecule, whereas the presence of quaternary amine nitrogens, polyhydroxyl groups and carboxylic acid functionality adds considerable hydrophilic character. In consideration of a suitable buffer for CZE separation of drug metabolites, the solubility of the drug and its metabolites in aqueous buffer systems are dependent upon their hydrophilic character. The hydrophobic character of HAL and the 10 synthetic standards of putative metabolites (see Fig. 1 for structures) prevented their solubilization in any of the conventional aqueous

buffers used in CE, such as phosphate, borate, or ammonium acetate. However, addition of 10% methanol was enough to solubilize all eleven compounds in a volatile run buffer comprising 50 m*M* ammonium acetate [8,49]. Furthermore, addition of organic modifiers, such as methanol, can also dramatically improve resolution of mixtures, since electroosmotic flow (EOF) is reduced by the increased buffer viscosity.

A perceived limitation of CZE is that it cannot separate neutrals since they possess no electrophoretic mobility. Indeed, some authors have questioned the limitations of CZE in the analysis of pharmaceutical agents [21,50], since most species are neutral. However, the majority of drugs and their subsequent metabolites possess functional groups that are either acidic (-COOH, -SH) or basic ($-NR_2$). Hence, modifying the pH of the buffer solution affects the charge (or partial charge) on the parent drug and its metabolites. In the case of HAL and the 10 synthetic standards, addition of acetic acid increases the acidity of the separation buffer (pH 4.1) and leads to protonation of basic nitrogens and results in cationic character for HAL, CPHP, HP^+,

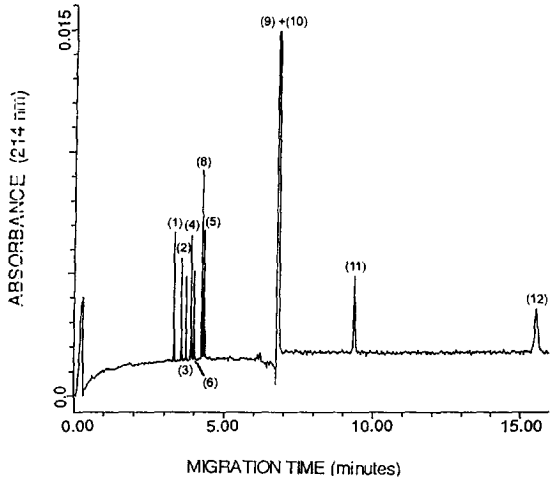

Fig. 2. CZE separation of HAL (4) and ten synthetic standards/putative metabolites (see Fig. 1 for numbering key). Analysis performed on a Beckman P/ACE 2100 CE, monitored at 214 nm. Separation buffer consisted of 50 m*M* NH_4OAc containing 10% MeOH and 1% AcOH (pH 4.1), run on an uncoated fused-silica capillary (57 cm×75 μm I.D.). Separation voltage 30 kV at 25°C, with a 1 s pressure injection.

HTP, RHAL, HNO and HTPNO. In addition, two compounds, FBPA and FAA, are relatively strong acids and, hence, were predominantly dissociated in this separation buffer solution, even at pH 4.1, and detected as anions. Only two compounds, FBPOH and FBALD, co-migrated under these conditions since the functional groups of these species were not ionized at pH 4.1 and both moved with the EOF. These results are shown in Fig. 2 for HAL and 10 synthetic standards using a CZE run buffer of 50 m*M* ammonium acetate containing methanol (10%) and acetic acid (1%).

3.1.1. Organic modifiers and non-aqueous-CZE

A variety of organic modifiers including methanol, isopropanol, acetonitrile, tetrahydrofuran and formamide [40,46], have been used in the CZE separation of small organic molecules. In CZE, increasing the methanol concentration causes an increase in the viscosity of the separation buffer and a concomitant decrease in EOF [46]. However, increasing acetonitrile concentration does not significantly alter viscosity or EOF velocity. Therefore, careful consideration of the selection of a suitable organic modifier is necessary.

It has been claimed that CZE migration times are erratic when buffers containing >40% organic solvent are used and this leads to electrical breakdown [51]. However, as has been previously reported, the CZE separation of small organic molecule mixtures can be readily and reproducibly achieved using nonaqueous separation media [40,45,46,52]. Typically, a nonaqueous electrolytic medium is prepared by dissolving a volatile salt (e.g., NH_4OAc) in an organic solvent (e.g., MeOH). Solution acidity is modified by addition of an organic acid (e.g., acetic or formic acid) or base (e.g., NH_4OH). Such media, once prepared, are stored at −20°C until further use to prevent reactions of acid/base with the organic solvent.

The consequences of using a nonaqueous separation medium are demonstrated in Fig. 3 for a mixture of HAL and six synthetic analogues. Using a separation buffer consisting of 5 m*M* NH_4OAc containing 50% MeOH with 1% AcOH, only partial resolution is observed (Fig. 3A). However, resolution of all seven structurally similar components is seen

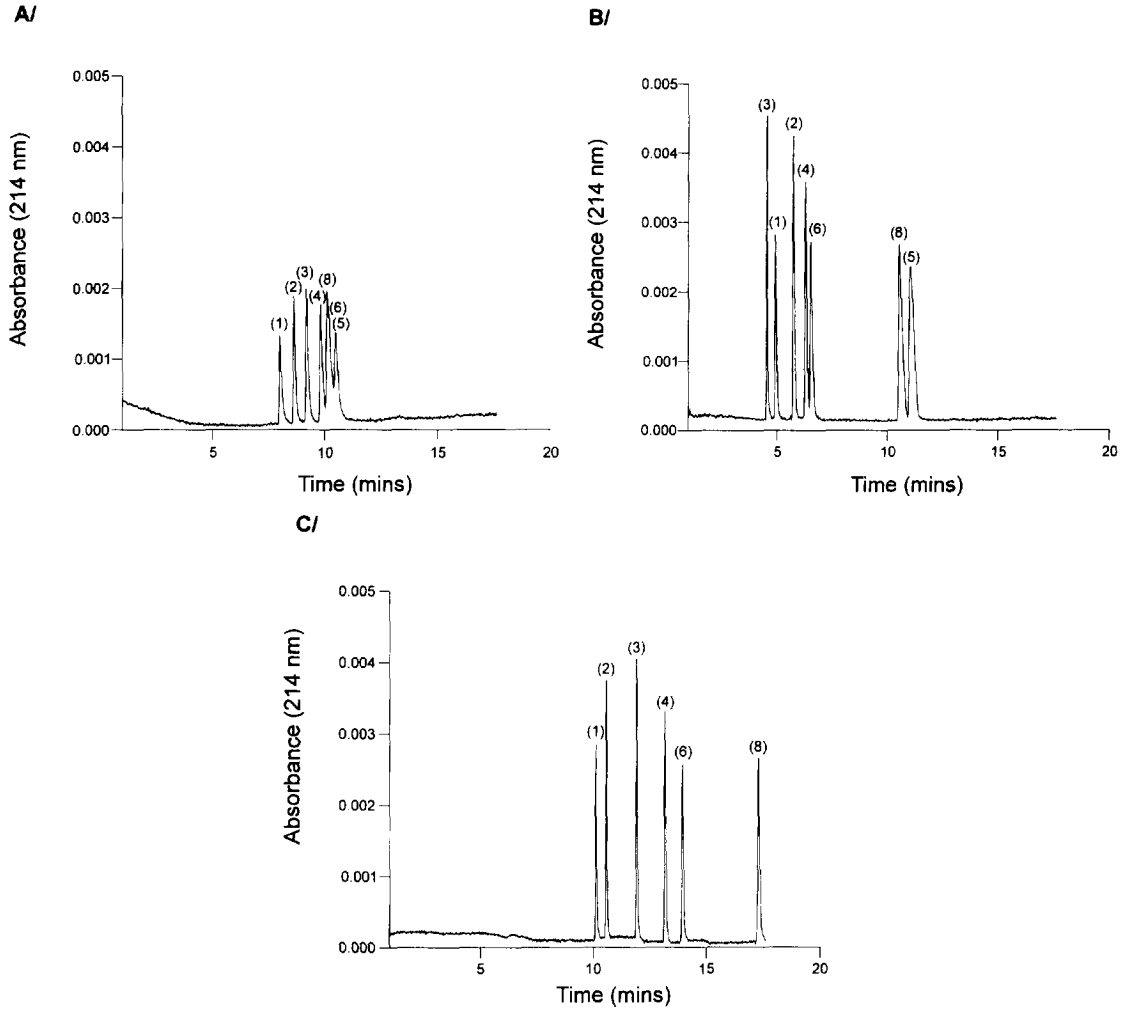

Fig. 3. CZE separation of HAL and six synthetic analogs, namely CPHP, HP^+, HTP, HTPNO, RHAL, and HNO (see Fig. 1 for numbering key). Analysis performed on a Beckman P/ACE 2100 CE, monitored at 214 nm, using an uncoated fused-silica capillary (57 cm×50 μm I.D.) at 30 kV and 25°C. Separation buffer conditions were as follows: (A) 5 m*M* NH_4OAc–50% MeOH–1% AcOH; (B) 5 m*M* NH_4OAc–100% MeOH–1% AcOH; (C) 50 m*M* NH_4OAc–50% MeOH–1% AcOH. It should be noted that for comparison purposes the electropherogram is shown for 0–20 min. However, for Fig. 3C the seventh component HTPNO (8) had a migration time of ~24 min and is not shown.

when 5 m*M* NH_4OAc dissolved in 100% MeOH containing 1% acetic acid is used as the separation medium (Fig. 3B). Furthermore, use of the nonaqueous separation medium results in a reduction of analyte–wall interactions leading to a decrease in peak width and peak tailing, as well as a significant increase (>100%) in analyte recovery. This is due to a reduction in loss of analytes onto the large surface area of the capillary wall.

3.1.2. Ionic strength

High-ionic strength buffers (>100 m*M*) lead to suppression of analyte–analyte and analyte–wall interactions [8,52]. However, such separation buffers can also lead to significantly increased Joule heating which results in loss of resolution and may lead to analyte instability. Also, as ionic strength is increased, a concomitant decrease in zeta potential is observed. This results in a decrease of EOF in direct

proportion to the square root of buffer concentration. Provided Joule heating effects are not significant, increasing the ionic strength of the separation buffer leads to enhanced resolution as the analytes are on the capillary much longer as they migrate slower. This can be seen in Fig. 3 for the mixture of HAL and the six synthetic standards. By increasing the NH_4OAc concentration from 5 m*M* (Fig. 3A) to 50 m*M* (Fig. 3C), peak widths are much sharper and resolution is enhanced compared to using 5 m*M* NH_4OAc in 100% MeOH (Fig. 3B). It should be noted that it was not possible to obtain a steady CE current using 50 m*M* NH_4OAc in 100% MeOH [52].

3.1.3. pH of CZE separation buffer

Salomon [53] previously reported that an optimal CZE separation of seven tricyclic amine antidepressant drugs was achieved when the pH of the separation buffer was close to the pK_a of the secondary or tertiary amine functional group present in these compounds. If the pH was selected above the pK_a for these bases, separation was not possible. This concurs with our previous report that subtle manipulation of pH can bring about separation of structurally similar compounds [8,49]. However, it is important to understand that the pH of the CZE separation buffer also influences the degree of protonation of silanol groups on an uncoated capillary wall, as well as the chemical stability of analytes present.

In the case of HAL (pK_a~8.3) a pH of ~4.1 was sufficient to bring about adequate resolution of the eleven- (Fig. 2) or seven-component (Fig. 3B) mixture. This was brought about by minimizing analyte–wall interactions since most of the silanol groups (pK_a~6–9) of the capillary wall are protonated at this pH. Furthermore, by ensuring that the pH was not too acidic, no chemical modifications such as dehydration of HAL, RHAL, or HNO were observed.

3.2. CZE–DAD

In the analysis of unknown compounds such as drug metabolites any structural information is important. Therefore, having determined optimal CZE conditions for separating HAL metabolites, we evaluated the use of on-line CZE–DAD in investigating drug metabolites mixtures. The advent of CZE–DAD has only occurred recently [27] even though this detection system has been in routine use on HPLC instruments for over a decade. An advantage of DAD over single wavelength detection is that a complete UV spectrum of an analyte can be obtained. This is particularly useful in the case of drug metabolism studies since the parent drug usually has a characteristic UV spectrum, which is only slightly modified in functionality when subjected to biotransformation. Hence, CE coupled to DAD can be exceedingly useful for screening complex mixtures and tentatively identifying metabolites based on their CE migration time and UV spectrum when compared to synthetic standards. This approach has been used by a number of workers (see Table 2) and was pioneered by Thormann for the analysis of barbiturates [25] and tetrahydrocannibinol metabolites [26] in urine, and the subject was recently reviewed by Heiger et al. [27].

Initially, we investigated the use of CZE–DAD on a mixture of HAL and six synthetic analogs employing the CZE run buffer conditions of 50 m*M* ammonium acetate containing 10% methanol and 1% acetic acid, as described previously. It was possible to baseline resolve all seven compounds (as already shown in Fig. 3B) as well as acquire UV spectra on HAL (λ_{max} ~248 nm, shoulder at 218 nm); CPHP (λ_{max} 218 nm); HP^+ (λ_{max} 298 nm, second maxima at λ 252 nm); HTP (λ_{max} 249 nm); RHAL (λ_{max} 220 nm); HNO (λ_{max} 248 nm); and HTPNO (λ_{max} 249 nm) [41].

Subsequently, analysis of a metabolite mixture derived from an in vitro guinea pig hepatic microsomal incubation by CZE–DAD revealed seven components (see Fig. 4). It was not possible to resolve all seven components (as was possible for the synthetic standards - see Fig. 3B), and this appears to be a feature related to biologically derived metabolites and could be due to dynamic range limitations when a large excess of unmetabolized parent drug is still present. The major component had a UV spectrum identical to unmetabolized parent drug HAL (Fig. 5A). Comparison of migration times (relative to that of HAL) and UV spectra of the other components in the electropherogram (Fig. 5A and Fig. 5B) to those of synthetic standards tentatively revealed the presence of CPHP, HP^+, HTP, and RHAL and FBPA. The relative migration time of the

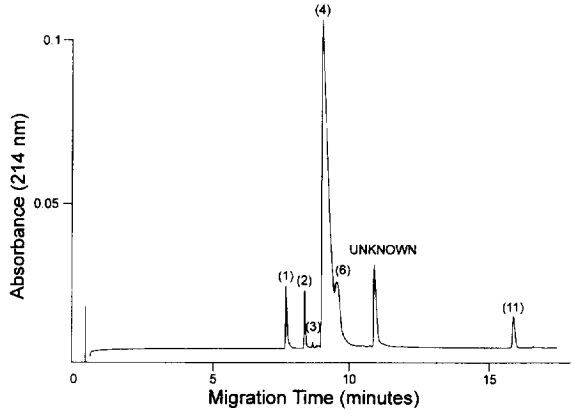

Fig. 4. CZE–DAD analysis of an in vitro guinea-pig hepatic microsomal incubation of HAL. Analysis performed on a modified Beckman P/ACE 5000 equipped with DAD. Separation conditions as for Fig. 2, except applied CE voltage was 20 kV (see Fig. 1 for numbering key).

seventh component (marked UNKNOWN in Fig. 4) was similar to HNO, but actually ~0.5 min slower. Furthermore, comparison of the UV spectra of HNO (λ_{max} 248) with the unknown (λ_{max} 246) revealed some differences (see Fig. 5C), confirming that a new, as yet unidentified, metabolite, was produced in the guinea-pig microsomal incubation (see later for further discussion).

In an attempt to evaluate CZE–DAD as a screening method for in vivo derived metabolites of HAL, a urine sample from a female patient receiving 0.5 mg/day of the parent drug was analyzed [41]. The urine sample was subjected to partial cleanup prior to analysis by CZE–DAD using the run buffer conditions described above. The electropherogram (shown in Fig. 6) was exceedingly complicated and contained numerous components not found in a "control" urine sample (results not shown). Based on the migration times of both synthetic standards of HAL and in vitro microsomal incubation derived metabolites, any phase I metabolites present in the urine should migrate in the ~3–10 min region of the electropherogram. Investigation of this region did not reveal any evidence of the presence of unmetabolized parent drug HAL or any of the expected phase I metabolites. This observation could be due to: (1) concentration levels of HAL or metabolites were below the detection limits of DAD. (2) co-migration

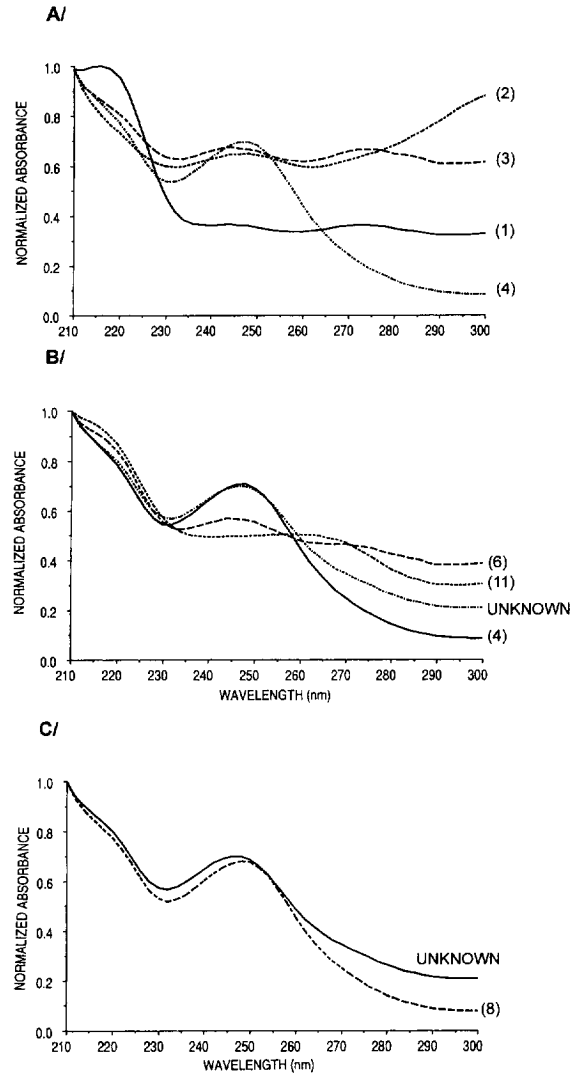

Fig. 5. UV spectra (200–300 nm) acquired by CZE–DAD of the guinea-pig microsomal incubation. Based on a comparison of migration times (relative to HAL) with synthetic standards tentative UV spectral assignments are shown. (A) CPHP (1), HP^+(2), HTP (3) and HAL (4) (shown for reference). (B) RHAL (6), FBPA (11) and an UNKNOWN. (C) A comparison of CZE–DAD of synthetic HNO (8) versus guinea-pig microsomal incubation derived UNKNOWN.

of HAL and/or metabolites with major contaminants may lead to a dynamic range problem in terms of detecting the minor HAL-derived components. These findings indicate that the use of CE–UV or CE–DAD has limited use in the structural characterization of

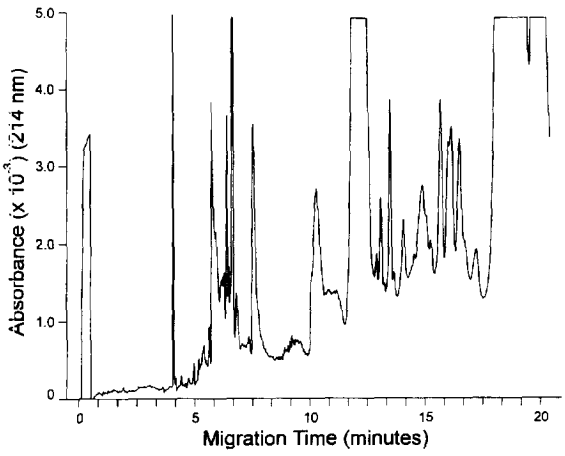

Fig. 6. CZE analysis of a urine sample obtained from a 27-year-old female taking 0.5 mg/day of HAL. Analysis was carried out on a Beckman P/ACE 2200 with DAD. Conditions as for Fig. 4.

new in vivo derived drug metabolites. This is due to the paucity of structural information one can obtain from the full UV spectrum. Therefore, we investigated the use of on-line CZE–MS in such studies.

3.3. On-line CZE–MS

A rapidly emerging approach to afford structural information of drug metabolites from complex mixtures is on-line CE–MS [54]. We and others have described in detail the usefulness of CE used in conjunction with ESI-MS [29–34,54]. Specifically, we replace the stainless steel spray needle in the ESI source with the CE capillary. We also use a coaxial sheath liquid, as previously described by Smith and co-workers [29,30] as the counter electrode for the CE capillary. This is all shown schematically in Fig. 7. Several practical aspects of CE–MS need to be considered, and they are listed below, as well as reported in detail elsewhere [54].

1. Good electrical contact is necessary at the outlet of the CE capillary in the mass spectrometer. This is achieved by removing the polyimide from the outlet end of the CE capillary and the use of a sheath liquid (e.g., isopropanol–water–acetic acid in a ratio of 60:40:1, v/v/v) as an effective means of maintaining electrical contact.

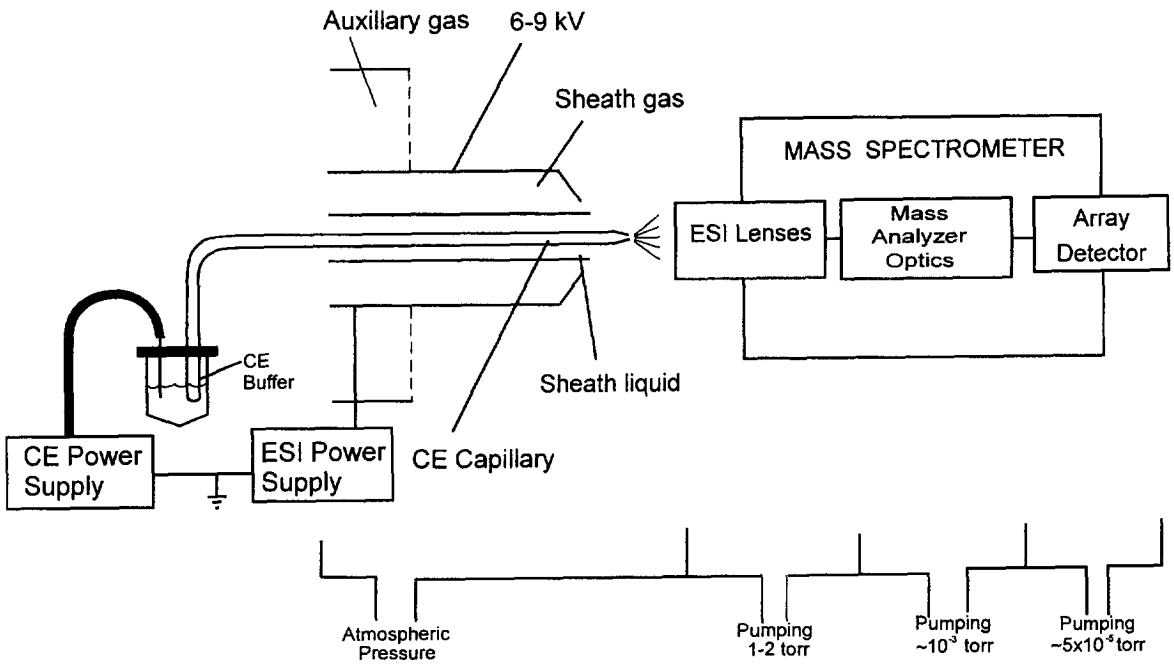

Fig. 7. Schematic of a CE–ESI-MS interface connected to a magnetic sector instrument. Note that it is not drawn to scale, since the ESI assembly is enlarged for the purposes of clarity.

2. Location of the CE capillary within the ion source is important since unstable ESI performance can often result from poor CE capillary positioning. Furthermore, shaping the capillary tip to a fine point can dramatically enhance sensitivity.
3. Use CE separation media of low ionic strength. Optimum CE–MS performance is achieved when CE and ESI currents are similar. High ionic strength CE buffers are not precluded, but reduced CE voltage may be required for stable ESI.
4. CE separation media to be prepared from volatile salts. Involatile buffers will be deposited within the ESI source and will eventually cause electrical breakdown.
5. ESI interfaces have spray configurations that are either grounded or maintained at high voltage (6–10 kV). We have noted no operational differences between these interfaces for CE–ESI-MS applications.

3.3.1. CZE–MS of synthetic standards

Initial CZE–ESI-MS studies were performed on HAL and the six synthetic analogs CPHP, HP^+, HTP, HTPNO, RHAL and HNO (see Fig. 1 for structures) [39,41]. The CZE separation buffer of 50 m*M* NH_4OAc–10% MeOH–1% AcOH was used to baseline resolve all seven components. This is shown as the CZE–ESI-MS ion electropherogram in Fig. 8.

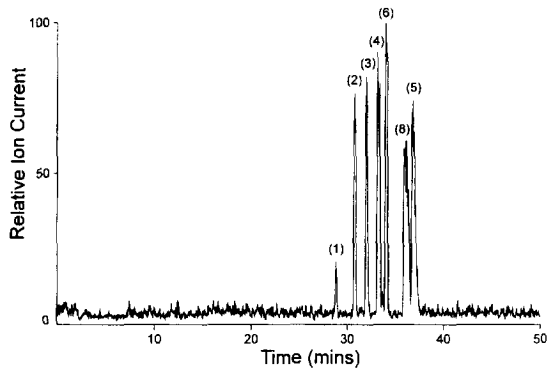

Fig. 8. CZE–MS analysis of HAL and six synthetic analogs (see Fig. 1 for numbering key). CZE separation was carried out on a Beckman P/ACE 2100 instrument using 50 m*M* NH_4OAc–10% MeOH–1% AcOH at 15 kV on an uncoated fused-silica capillary (65 cm×50 μm I.D.) using 30 s pressure injection. The sheath liquid for the ESI was isopropanol–water–AcOH (60:40:1, v/v/v) and the ESI voltage was −3.4 kV. The scan range was 425–125 at 3 s/decade at a resolution of ~1200.

The limits of detection of each of these components was ~10 fmol when using the PATRIC focal plane detector.

Structural information for each compound was generated using on-line CZE–skimmer CID-MS. In such experiments, the voltages of the capillary and tube lenses are varied while the first skimmer is maintained at accelerating voltage. Such conditions [55] lead to excitation of ions, and low energy collisions can occur in this high pressure region of the ESI source that result in the production of structurally significant product ions. An example of CZE–skimmer CID-MS is shown for the parent drug HAL in Fig. 9. The dominant ion at *m/z* 165 is reported to arise from charge-initiated fragmentation of the alkyl carbon-nitrogen bond with expulsion of the nitrogen containing moiety as a neutral species [56]. Other major product ions at *m/z* 358 and 123 as well as minor ions at *m/z* 206 and 194 are structurally assigned in both Table 3 and Fig. 9. CZE–skimmer CID-MS product ion data for all synthetic standards/putative metabolites of HAL are also summarized in Table 3. These data are similar to those reported for the same compounds when subjected to CID processes in a quadrupole collision cell of a hybrid instrument of EBQ_1Q_2 configuration [56]. However, CZE–skimmer CID-MS offers the advantage of detection of distinctive isotope contributions in product ion spectra. For example, the product ion at *m/z* 358 derived from HAL has an accompanying chlorine isotope contribution at *m/z* 360, signifying that this ion retains the chlorophenyl moiety. In contrast, product ions at *m/z* 165 and 123 do not show such accompanying isotope contribution indicating that charge retention is on the fluorophenyl portion of the molecule. A potential limitation of CZE–CID-MS is the requirement for analyte separation prior to entrance into the ESI source. Otherwise composite product ion spectra are obtained, making data interpretation difficult.

3.3.2. CZE–MS of guinea pig microsomal incubation

In order to more fully characterize the in vitro derived metabolites from the guinea-pig microsomal incubation of HAL, the mixture was subjected to both CZE–ESI-MS and CZE–ESI-CID-MS analysis. Analysis of the in vitro guinea pig hepatic micro-

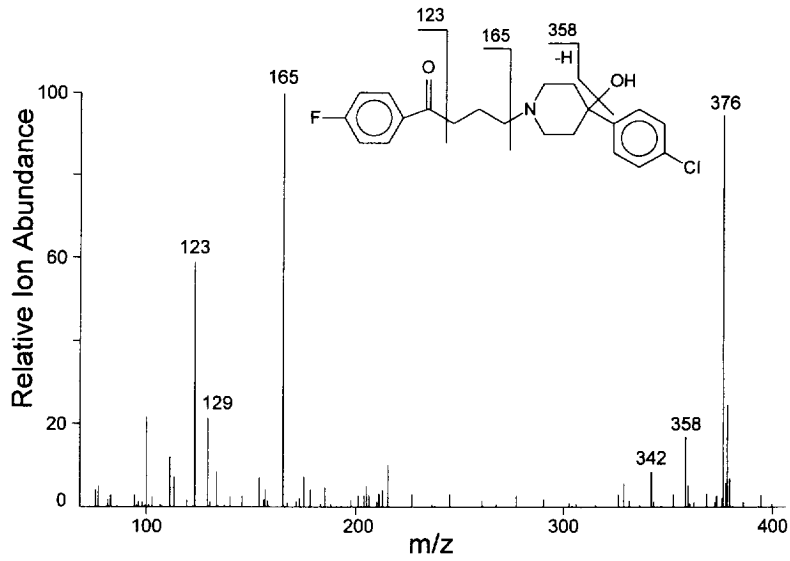

Fig. 9. CZE–ESI skimmer CID-MS spectrum of parent drug HAL ($MH^+ = 376$). Conditions as in Fig. 8, except scan range was 400–50 at 2 s/decade. Spectra were acquired with the PATRIC focal plane detector.

somal incubate by CZE–ESI-MS after solid-phase cleanup revealed prominent ions at *m/z* 212, 354, 376 and 392, corresponding to CPHP, HP^+, unmetabolized HAL and UNKNOWN, respectively (see Fig. 10). Under the CZE conditions used biologically derived RHAL appeared to co-migrate with HAL. This was discernible due to the appearance of a prominent ion at *m/z* 378 corresponding both to the chlorine isotope contribution of HAL, plus the protonated molecular species (MH^+) of RHAL. An ion at *m/z* 380 corresponding to the chlorine isotope contribution (^{37}Cl) of RHAL confirmed the presence of this metabolite. We believe that the cause of the co-migration of HAL and RHAL was sample-related since both compounds were baseline-resolved in the synthetic standard mixture (Fig. 8), but not in the guinea-pig microsomal incubation. The ion chromatogram derived from the on-line CZE–ESI-MS analysis of the guinea pig microsomal incubation mixture also revealed an ion at *m/z* 358. This ion can be obtained by facile dehydration of HAL under CE–ESI-CID-MS conditions in the ESI source, as noted in Table 3. However, closer inspection of the ion chromatogram revealed two temporally resolved ions at *m/z* 358 (Fig. 10). By comparison with the migration and ion chromatogram times of synthetic standards these two distinct ions can be assigned to HTP and HAL–water, respectively.

All molecular ions observed in the CZE–MS analysis of the microsomal mixture were subjected to CE–ESI-CID-MS and afforded product ions similar to those observed for synthetic standards, thereby determining the presence of CPHP, HP^+, unmetabolized HAL, RHAL, and HTP. A CZE–ESI-CID-MS spectrum of UNKNOWN at *m/z* 392 was almost identical to that obtained on the synthetic standard HNO (Table 3). Since the major ion of this product ion spectrum is *m/z* 165, which corresponds to $[F\text{-}C_6H_4\text{-}C(O)CH_2CH_2CH_2]^+$, it is apparent that the metabolic modification of HAL had occurred at the piperidine end of the molecule. However, the lack of fragmentation with charge retention on the substituted piperidine prevented full structural elucidation of this compound.

Ultimately both the standard HNO and UNKNOWN were subjected to CZE–FLS-MS–MS. In this instance, the product ions at $MH^+ = 392$ are subjected to high energy (5 kV) collisions with helium in the first field free region. The resulting product ions are then analyzed by maintaining a constant *B/E* ratio and detected at the PATRIC detector. Some discernible differences were observed between the product ion spectrum of synthetic HNO

Table 3
CZE–skimmer CID-MS–MS product ion data for synthetic standards of HAL

Ions detected (m/z)	Relative ion abundance (%)	Product ion assignment
HAL		
376	6	MH^+
358	2	$[MH\text{-}H_2O]^+$
206	<1	$[CH_2{=}NC_5H_7\text{-}C_6H_4Cl]^+$
194	<1	$[HNC_5H_7\text{-}C_6H_4Cl + H]^+$
165	100	$[F\text{-}C_{10}H_{10}O]^+$
123	31	$[F\text{-}C_6H_4\text{-}C{=}O]^+$
CPHP		
212	100	MH^+
194	94	$[MH\text{-}H_2O]^+$
HP$^+$		
354	10	M^+
165	98	$[F\text{-}C_{10}H_{10}O]^+$
123	100	$[F\text{-}C_6H_4\text{-}C{=}O]^+$
HTP		
358	22	MH^+
194	19	$[HNC_5H_7\text{-}C_6H_4Cl + H]^+$
165	100	$[F\text{-}C_{10}H_{10}O]^+$
123	22	$[F\text{-}C_6H_4\text{-}C{=}O]^+$
HTPNO		
374	17	MH^+
165	100	$[F\text{-}C_{10}H_{10}O]^+$
123	20	$[F\text{-}C_6H_4\text{-}C{=}O]^+$
RHAL		
378	17	MH^+
360	35	$[F\text{-}C_{10}H_{10}O]^+$
149	60	$[F\text{-}C_6H_4\text{-}C{=}O]^+$
HNO		
392	8	MH^+
165	100	$[F\text{-}C_{10}H_{10}O]^+$
123	18	$[F\text{-}C_6H_4\text{-}C{=}O]^+$

Note that the relative ion abundance data are highly dependent upon skimmer, capillary and tube lens voltage. These data were acquired at skimmer, capillary and tube voltages of 5 kV, 100 V and 212 V, respectively.

and UNKNOWN. Most obvious was that the product ion abundance of m/z 224 and 206 were significantly reduced for UNKNOWN (Fig. 11) compared to HNO. This tentatively indicates that it is unlikely that UNKNOWN is an N-oxide but that oxidation has occurred somewhere else at the piperidine end of the molecule. Based on the CZE–DAD data, it is unlikely that oxidation of the chlorophenyl ring has occurred, since the UV of UNKNOWN (Fig. 5C) has

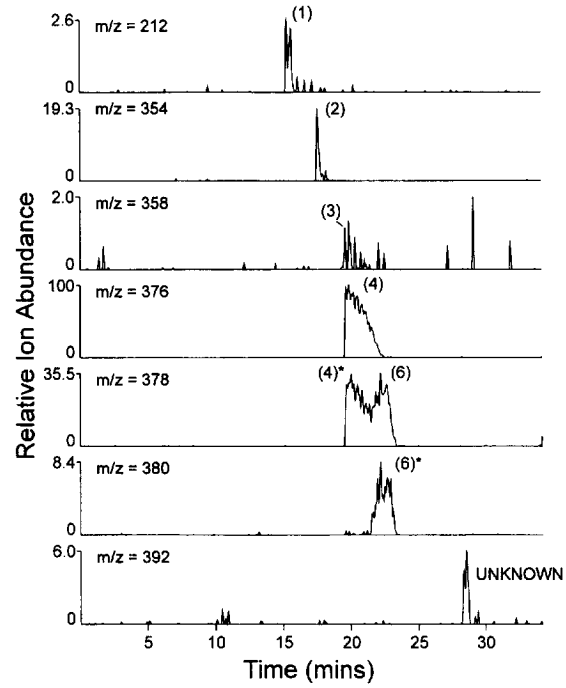

Fig. 10. CZE–ESI-MS analysis of the in vitro guinea-pig microsomal incubation of HAL after off-line solid-phase extraction. Conditions as for Fig. 8 (see Fig. 1 for numbering key). Note that (4)* and (6)* denote the ^{37}Cl isotope molecular ions of HAL and RHAL, respectively.

a λ_{max} of ~245 nm compared to HNO λ_{max} of ~250 nm. This hypsochromic shift indicates that the chlorophenyl ring is not modified. Based on these facts, a possible structure for UNKNOWN is shown in Fig. 11 and corresponds to oxidation of the piperidine ring to afford the hydroxy piperidine moiety.

3.3.3. CZE–MS analysis of human urine

Previously we had noted that it was not possible to detect any unmetabolized HAL or its metabolites in urine using CZE–DAD. Therefore, we attempted the analysis of the same urine, obtained from a female patient receiving 0.5 mg/day of HAL, using CZE–MS. In order to maximize detection sensitivity, we employed the PATRIC detector in static mode. In this case the detector was centered at m/z 365 with an 8% mass window, without scanning. Hence although the mass range was limited (m/z 380–350), it afforded minimum loss of ions since no scanning

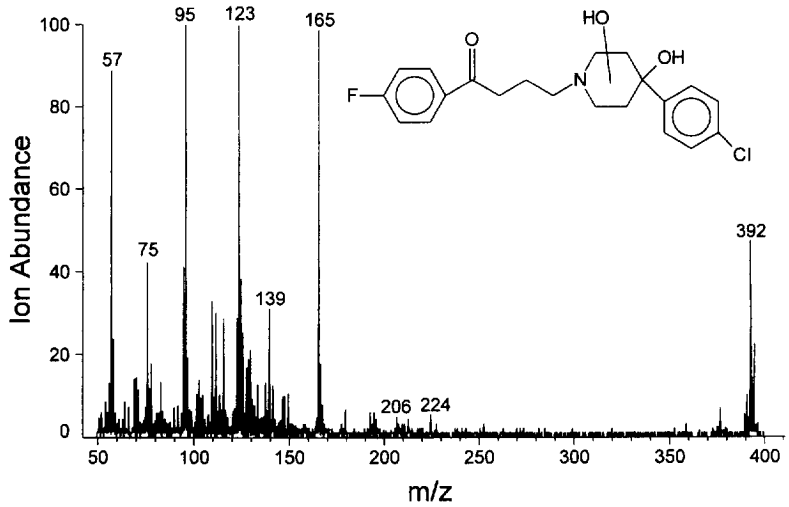

Fig. 11. CZE–FLS-MS–MS analysis of the unknown metabolite ($MH^+ = 392$) derived from the in vitro guinea-pig microsomal incubation. $MH^+ = 392$ was used as the link mass and helium (40% attenuation) was used as the collision gas. A 2% PATRIC window was used.

of the magnet occurred. It was possible using this approach and employing optimal CZE buffer conditions (described previously) to detect ~100 attomol of HAL standard.

The urine sample which had been subjected to off-line concentration on a C_{18} Sep-Pak was injected onto the CE capillary using transient isotachophoresis (tITP) conditions [8,45] for 20 s. This resulted in ~200 nl of the original 10 μl concentrate (see Experimental) being introduced onto the CE capillary. Subsequently the CE voltage was applied and analytes were subjected to CZE–MS analysis. An ion at m/z 376 corresponding to the protonated molecular mass (MH^+) of unmetabolized HAL was clearly detected, as shown in Fig. 12. Furthermore, the abundance of the ion at m/z 378 was much greater than the ~30% predicted for the chlorine isotope contribution of HAL. An ion chromatogram at m/z 380 corresponding to the chlorine isotope contribution of RHAL was also clearly detectable (Fig. 12). In addition, the migration time was slightly later than for HAL, strongly suggesting the presence of RHAL. No other metabolites of HAL were detected. Hence even employing static PATRIC detection coupled with off-line solid-phase extraction it was only possible to detect a single known metabolite of HAL. This indicated:

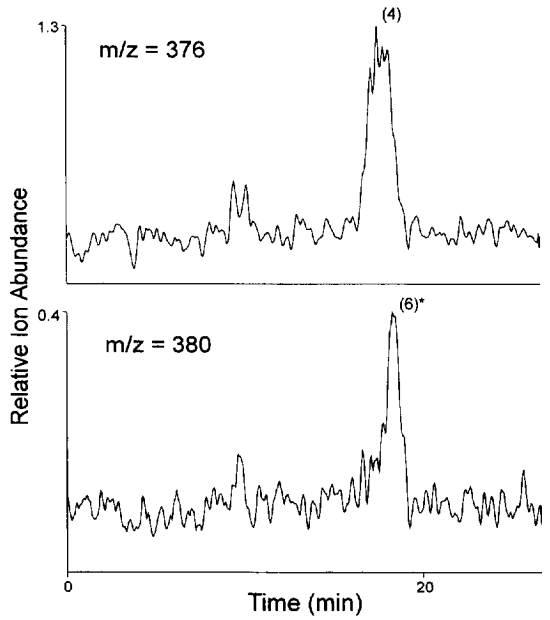

Fig. 12. CZE–ESI-MS analysis of a solid-phase extraction of 50 μl of urine from a patient treated with 0.5 mg/day of HAL. Conditions identical to Fig. 8; however, the PATRIC detector was employed in 'static' mode centered at m/z 365 using an 8% mass range window. Only ions at m/z 376, 378 (not shown) and 380 corresponding to the molecular ions of HAL, RHAL and [^{37}Cl]RHAL were detected.

1. Sample losses due to off-line concentration appeared to be considerable.
2. Static PATRIC detection did not allow detection of metabolites outside the 8% mass range.
3. The limited loading capacity of conventional CE capillaries, even using stacking or focusing injection techniques (e.g., tITP), was insufficient for the analysis of low concentrations of analytes derived from human body fluids such as urine.

3.4. mPC–CE and mPC–CE–MS

3.4.1. mPC–CE

In order to overcome the problems associated with the analysis of in vivo derived metabolites, as well as the more general problem of poor concentration limits of detection (CLOD) associated with conventional CE, we developed mPC–CE. We have described this technology and its development elsewhere [40,57–64]. A schematic of the mPC–CE cartridge is shown in Fig. 13. For convenience, the membrane was installed in a PTFE cartridge and installed at the inlet end of the conventional CE capillary. This ensured that the mPC–CE cartridge could readily be removed to enable CE capillary cleaning and/or conditioning prior to mPC–CE analysis. Typically the analytes are loaded onto the mPC–CE cartridge in buffer/solvent that has less affinity than the adsorptive properties of the impregnated membrane. After the sample has been loaded, in our case by pressure injection, the membrane is washed with separation buffer to remove any salts, resulting in on-line sample cleanup, as well as concomitant conditioning of the CE capillary. Ultimately, analytes are eluted from the membrane with a minimum of organic solvent and subsequently the CE voltage is applied.

Attempts at loading high volumes of analyte solutions using mPC–CE resulted in some compromised CE performance, highlighted by slight peak broadening [65]. However, by employing mPC–CE in conjunction with the appropriate CE capillary chemistry such as analyte stacking or tITP after analytes were eluted from the membrane [65], no compromise in performance occurred. When small organic molecules such as drug metabolites are eluted from the membrane in organic solvent (e.g., MeOH) analyte stacking occurs when the CE voltage is applied (see Fig. 14). This is due to the fact that analytes are now in a low conductivity solution relative to the separation buffer which leads to a high field strength across the organic zone. This results in a rapid migration of analytes through the organic solvent which ultimately leads to localized stacking (Fig. 14).

The high loading capacity of the mPC–CE cartridge, as well as the lack of compromised CE performance when performed in conjunction with analyte stacking is demonstrated in Fig. 15. In this example, a mixture of HAL, RHAL, HP^+, HTP,

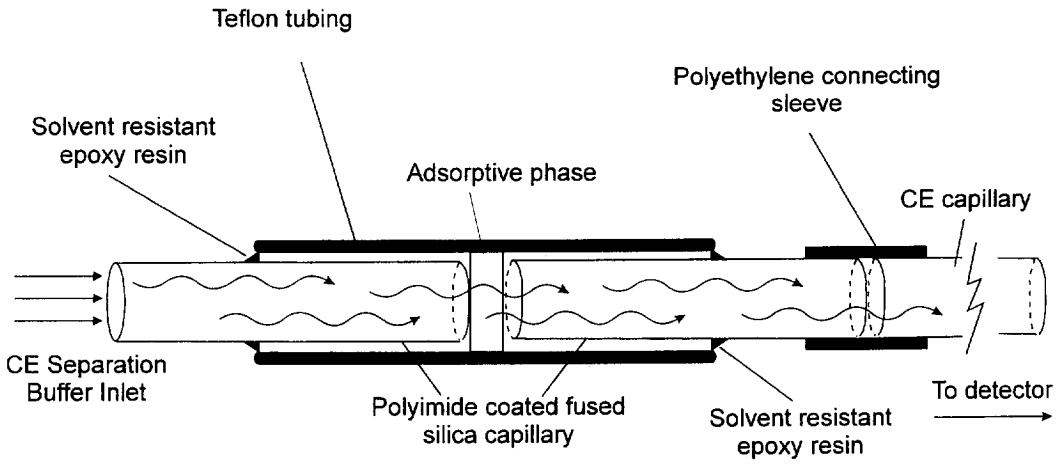

Fig. 13. Schematic (not to scale) of a mPC–CE cartridge. This is connected to a conventional CE capillary at the inlet.

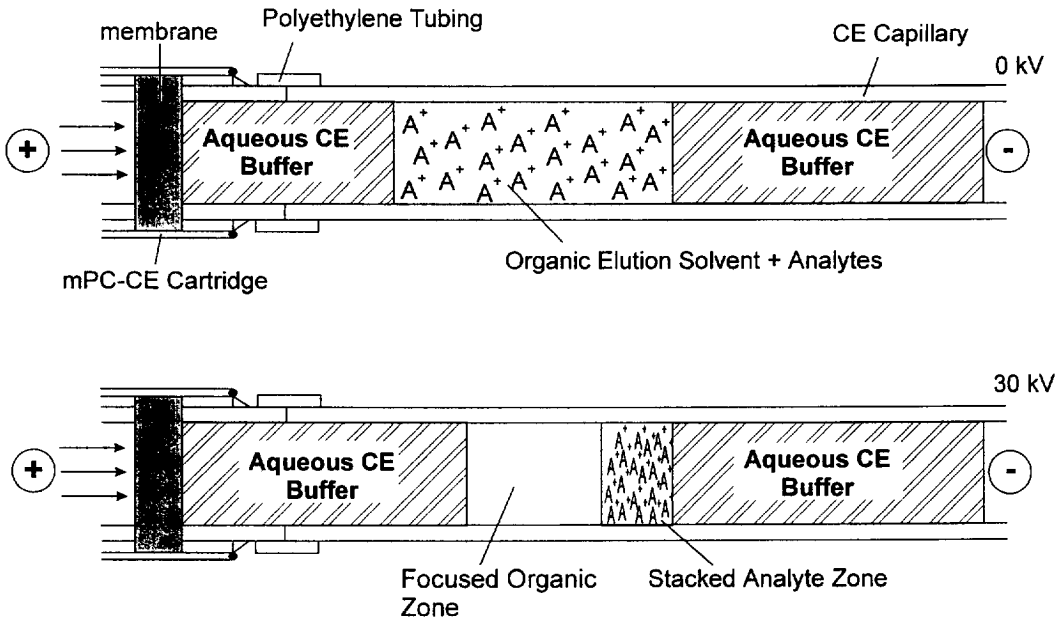

Fig. 14. Schematic of analyte stacking of compounds for use in conjunction with mPC–CE after analytes have been eluted from the membrane and the CE voltage applied.

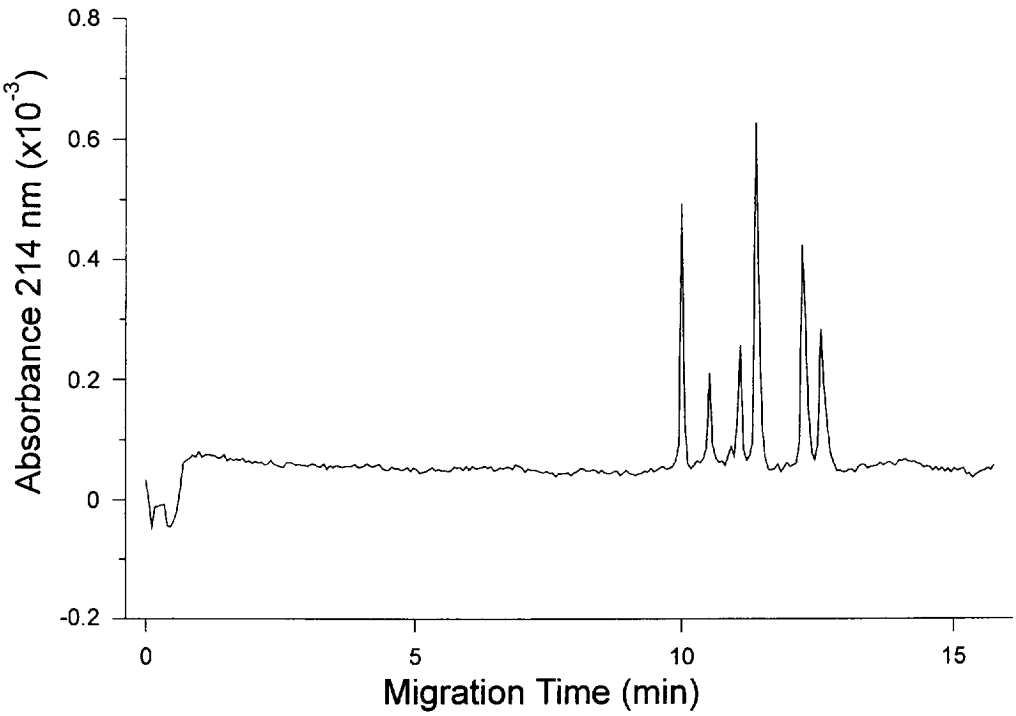

Fig. 15. mPC–CE analysis of 55 μl of a mixture of HAL, RHAL, HP^+, HTP, HTPNO and HNO at a concentration of 3.3 ng/ml. Analytes were eluted from the membrane with 50 nl of MeOH–CH_3CN (1:1), and subjected to analyte stacking and CZE in 50 m*M* NH_4OAc–10% MeOH–1% AcOH on application of CE voltage at 25 kV. Other conditions as for Fig. 3.

HTPNO and HNO were dissolved in the minimum of MeOH then diluted with separation buffer (50 m*M* NH_4OAc–10% MeOH–1% AcOH) to give a final concentration of 3.3 ng/ml. An aliquot of 55 μl of this solution was applied to the mPC–CE cartridge with pressure injection. The mPC–CE capillary was then flushed with CE separation buffer prior to elution of analytes from the membrane with a solution containing acetonitrile–methanol (1:1, v/v). Subsequently, analyte separation was completed by electrophoresis with component detection by UV at 214 nm. In this example, all components were baseline resolved. Furthermore, the amount of sample volume analyzed was more than three times greater than could be analyzed by conventional free solution capillary electrophoresis.

3.4.2. mPC–CE–MS and mPC–CE–MS–MS of human urine metabolites

The ultimate goal in drug metabolism studies is to determine in vivo metabolic pathways. In the case of HAL, although it is widely used as a neuroleptic agent it can also induce debilitating side-effects including Parkinsonian-like symptoms [1]. Previously, we described the use of CZE–ESI-MS with static PATRIC ion array detection for the analysis of HAL metabolites in urine. However, off-line solid-phase extraction and concentration were only sufficient to allow detection of RHAL. Therefore, we investigated the use of on-line mPC–CE–MS for the analysis of the same urine sample.

In this case, the only sample pretreatment undertaken was the addition of $ZnSO_4$ followed by centrifugation to remove precipitated urinary proteins. mPC–CE–MS was then used to load and analyze 10 μl of the resultant supernatant solution. Subsequently, $ZnSO_4$ and other hydrophilic sample contaminants were removed by washing the mPC–CE capillary with separation buffer (50 m*M* NH_4OAc–10% methanol–1% acetic acid). Analytes were subsequently eluted off the membrane into the CE capillary with 100% methanol (80 nl) and voltage applied across the CE capillary. Since analytes were in an organic solvent zone, analyte stacking was induced at the start of electrophoresis (shown schematically in Fig. 14). Membrane-PC–CE–MS analysis of the urine sample, employing full scan (125–450 u) mass detection, revealed ions at *m/z* 376.4, 380.3, 354.3, and 356.3 all of which exhibited a ^{37}Cl isotope contribution, tentatively identified as unmetabolized parent drug HAL, the known metabolite RHAL, and two pyridinium species HP^+ and RHP^+, respectively (Fig. 16). The presence of the metabolites was confirmed by using mPC–CE–MS–MS in the linked scan mode. 20 μl of patient urine was subjected to the same processing and analysis as described above, but each molecular ion detected was subjected to CID and the resulting product ions interpreted for structural information. A representative product ion spectrum of the HP^+ metabolite where $M^+=354$ was subjected to high energy collision is shown in Fig. 17. Although the abundance of product ions produced is limited, the data was comparable to linked scan MS–MS data obtained on synthetic standards (data not shown) as well as that shown in Table 3 for CZE–ESI-CID-MS–MS analysis. This confirms the presence of the metabolites RHAL, HP^+ and RHP^+. These results are consistent with the recently published work of Eyles et al. [66] and Rollema et al. [67] who reported finding HP^+ and RHP^+ present in both humans and

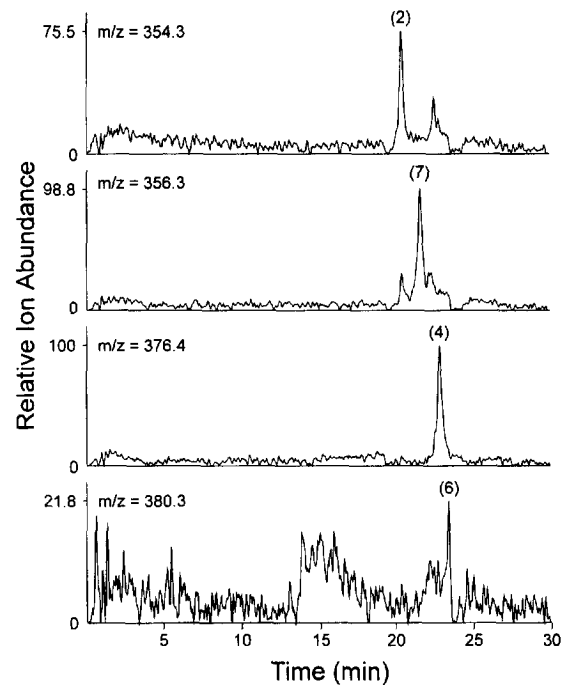

Fig. 16. mPC–CE–MS analysis of 10 μl of urine from a female patient receiving 0.5 mg/day of HAL. Conditions as for Fig. 15.

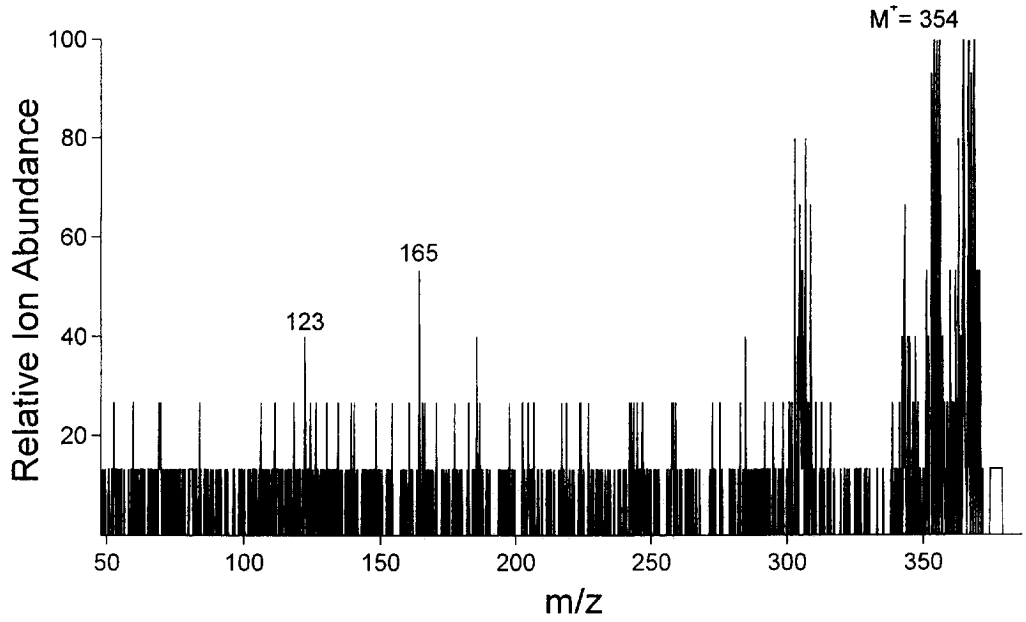

Fig. 17. mPC–CE–FLS-MS–MS of $M^+ = 354$ (HP^{-+}), product ion spectrum obtained from the analysis of 20 μl of urine. Conditions as for Fig. 15.

animals. The presence of the two pyridinium metabolites is indicative that HAL undergoes metabolic conversion via an active intermediate that is similar in nature to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) metabolism. MPTP is a known and powerful inducer of Parkinsonian-like symptoms in both animals and humans [68].

4. Conclusions

Understanding of the events that regulate both biological and physical properties of therapeutic agents requires the formidable task of isolation and characterization of metabolically transformed products. This task is further complicated by the fact that such compounds are present as minor components of extremely complex biological matrices. In this regard, the rapid, high-resolution separations and ease of method development afforded by CE have considerable attraction. Furthermore, the coupling of CE with DAD leads, potentially, to an effective means of screening biological matrices for the presence of drug metabolites. Ultimately the combination of CE with MS and tandem MS techniques yields structurally significant data from which metabolites may be characterized. However, these techniques are not without limitation. In particular, the dimensions of the CE capillary lead to rather a poor CLOD when compared to HPLC. Indeed, the typical injection volumes of CE (<50 nl) are in stark contrast to those of HPLC which can be in excess of 50 μl. For this reason, the techniques of mPC–CE and mPC–CE–MS have been developed. These more modern CE methods enable the introduction of sample volumes into the CE capillary that are comparable with and may often exceed those applied to a capillary HPLC column. Furthermore, mPC–CE(MS) technology may be used to pre-elute components of biological matrices (such as salts) that would otherwise influence, detrimentally, the electrophoretic separation of analytes. Another attribute of mPC–CE(MS) is that sample handling is reduced and this often results in increased recovery of compounds of interest. In addition, the exposure of the analyst to biological fluid and previously unidentified potentially toxic analytes is reduced.

Finally, from our investigations of HAL metabolism, we suggest that CE combined with DAD and, in particular MS, has the potential of revolutionizing

drug metabolism research. Furthermore, from our simple model of considering hydrophobicity (its solubility in aqueous solutions) and ionizable character at various pH conditions, development of generic conditions that enable the study of metabolic transformation of a variety of therapeutic agents is plausible and increases the attractiveness of CE for such research.

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