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Investigation of the metabolic fate of the neuroleptic drug haloperidol by capillary electrophoresis–electrospray ionization mass spectrometry

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ABSTRACT

Combined capillary electrophoresis–mass spectrometry (CE–MS) is a powerful analytical tool for the characterization of components of complex mixtures. Furthermore, when interfaced with an electrospray ionization (ESI) source, skimmer induced fragmentation can yield structural information that aids compound identification. We show that these techniques can be used to probe the metabolic fate of drugs by investigation of the phase I metabolism of the widely used neuroleptic drug haloperidol. In addition, we demonstrate differences of *in vitro* metabolism of haloperidol by mouse and guinea pig hepatic microsomes.

INTRODUCTION

The clinically proven neuroleptic agent haloperidol (HAL) belongs to the butyrophenone class of drugs and is used to treat psychotic disorders, such as schizophrenia [1]. It is also used in the treatment of hyperexcitable children, as well as to control the symptoms of Tourette's syndrome [1]. Research has shown that use of this drug can sometimes induce severe side effects (*e.g.* acute dystonic reactions, akasthisia, tardive dyskinesia) along with Parkinsonian-like symptoms [1–3].

HAL is widely used in clinical medicine; however, little is known about physiological factors that influence its metabolism or, indeed, correct therapeutic dosage. Recently, we and others have used modern analytical techniques, such as reversed-phase HPLC, tandem mass-spectrometry (MS–MS), and on-line HPLC–MS–MS, to investigate the metabolism of HAL [4–11]. Also, we have demonstrated the use of free solution capillary electrophoresis (CZE) for the analysis of HAL phase I metabolism [12,13]. A number of studies have shown that HAL is metabolised via one pathway, which leads to products that are structurally similar to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an agent known to induce Parkinsonian symptoms [14–16]. It has been suggested that structural functionality common to both compounds, HAL and MPTP, is responsible for inducing such effects [8,10,17].

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Isolation and structural characterization of metabolites is of paramount importance in drug metabolism studies. In this regard, the highly efficient separations afforded by capillary electrophoresis (CE) yields obvious advantages over classical separation techniques (*e.g.* HPLC) for resolution of chemically similar drug metabolites [12]. Furthermore, the development of on-line CE–MS, pioneered by Smith *et al.* [18–22] and Henion and coworkers [23–26], considerably enhances the utility of CE in the analysis of complex drug metabolism mixtures. However, to date there has been only one report on the use of CE–MS in drug metabolism structure elucidation. In this study, Johansson *et al.* investigated the separation and mass spectral analysis of a series of sulfonamide and benzodiazepines, in addition to the *in vivo* metabolism of fluorazepam [27].

In the present study, we have utilized on-line free solution CE coupled to ESI (CZE–ESI–MS), employing the coaxial sheath liquid methodology developed by Smith *et al.* [18–22], to investigate the phase I derived metabolites of HAL. We show differences in the metabolism of this drug by analysis of extracts derived from *in vitro* mouse and guinea pig hepatic microsomal incubates. In addition, we demonstrate the usefulness of variable skimmer voltage collision induced dissociation (skimmer CID) in the ESI source to aid in the structural characterization of drug metabolites.

EXPERIMENTAL

Chemicals

Haloperidol [HAL, 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol], potassium phosphate (monobasic), and zinc sulphate were obtained from Sigma (St. Louis, MO, USA). Gold grade ammonium acetate, glacial acetic acid, and magnesium chloride were obtained from Aldrich (Milwaukee, WI, USA). NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). HPLC grade solvents methanol and methylene chloride were obtained from Baxter (Minneapolis, MN, USA). High purity water was prepared in-house using a Sybron Barnstead PCS water purifier system (ex-Millipore) supplied by VWR (Minneapolis, MN, 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), and 4-fluorobenzoyl propionic acid (FBPA) were synthesized as described previously [11].

Capillary electrophoresis

Capillary electrophoresis separations were performed using a modified Beckman P/ACE 2100 Model CE (Fullerton, CA, USA), coupled to a Reason Technology 486 PC with control by System Gold software (Beckman). An uncoated capillary (either 65 cm × 50 μm I.D. or 100 cm × 50 μm I.D.) purchased from Beckman Instruments was used throughout. Prior to its use, the capillary was rinsed with 0.1 *M* sodium hydroxide (20 column volumes), water (20 column volumes), and buffer (10 column volumes). Between analyses, the capillary was washed with run buffer (10 column volumes). The buffer used to afford optimum separation of metabolites was 50 m*M* ammonium acetate containing 10% methanol and 1% glacial acetic acid in water at pH 4.1. Synthetic standards were individually dissolved in methanol (1 mg/ml) and 5 μl was removed from each vial and mixed in a single, clean vial to give a mixture containing HAL plus CPHP, HP^+, HTP, RHAL, HNO, HTPNO, and FBPA. The mixture was introduced by pressure injection (15 s), and all experiments were conducted with an applied voltage of 15 kV and a capillary temperature maintained at 25°C. Analyte detection was by mass spectrometry.

Mass spectrometry

All analyses were carried out on a Finnigan

MAT 900 mass spectrometer (Bremen, Germany) of EB configuration (where E is an electric sector and B is the magnet) with a PATRIC focal plane detector. An Analytica electrospray ion source (Analytica, Connecticut, USA) was used in a positive ion mode throughout, with the needle assembly at ground potential. The sample needle of the ESI source was replaced by the CE capillary from which 2–3 mm of the polyamide 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 CZE–MS runs, the scan range was 125–450 a.m.u. (exponential magnet scan from low to high mass) at a rate of 3 s/decade, whilst CZE–skimmer CID–MS experiments were performed with a scan range of 60–450 a.m.u. at a rate of 2 s/decade. An instrument resolution of ~1200 was employed throughout.

Microsomal incubations

English short-hair male guinea pigs were obtained from the Charles River Co. (Montreal, Canada). Animals were fasted overnight before sacrifice. Hepatic microsomal preparations were prepared using the centrifugation method described previously [28].

Mouse hepatic microsomes were prepared by differential centrifugation of freshly prepared liver homogenates from male $CD2F_1$ mice obtained from the National Cancer Institute (Bethesda,

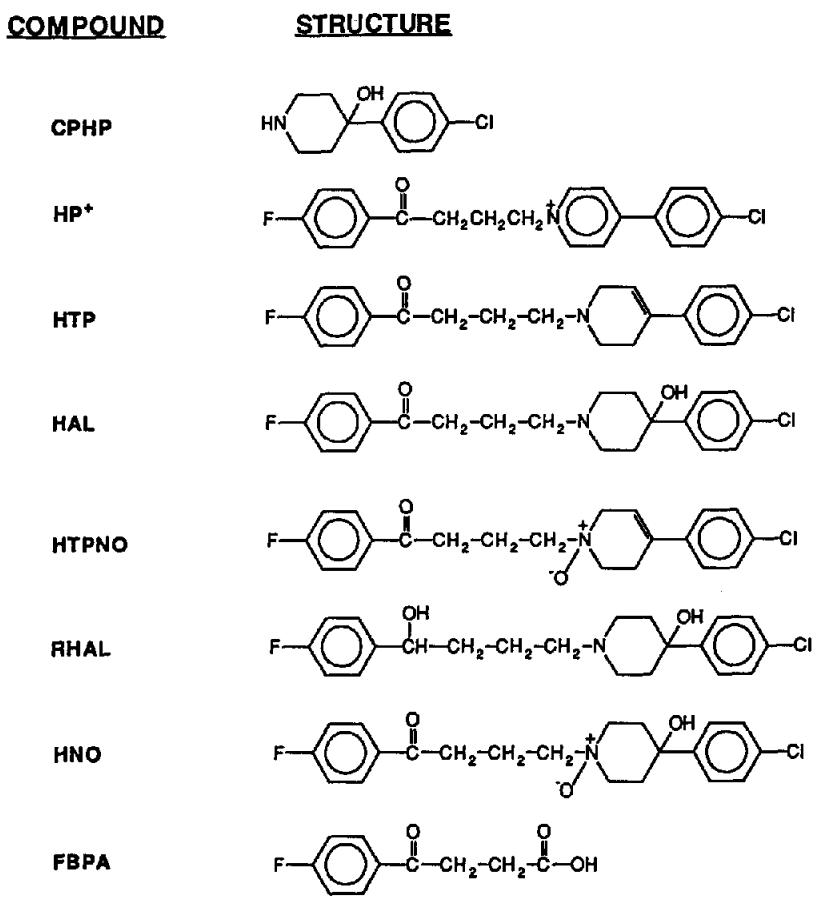

Fig. 1. Structures of putative phase I metabolites of HAL.

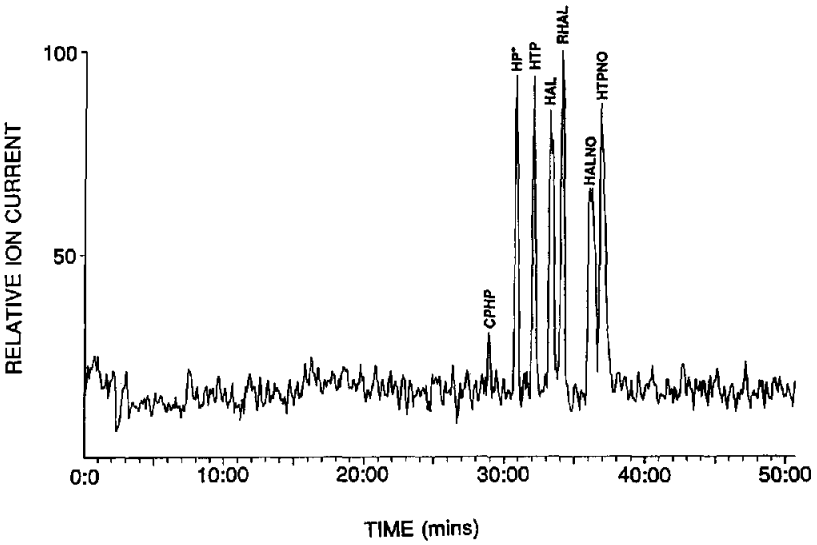

Fig. 2. A total ion-current electropherogram of a mixture of synthetic standards of putative phase I metabolites of HAL consisting of CPHP (MH^+ = 212), HP^+ (M^+ = 354), HTP (MH^+ = 358), unmetabolised HAL (MH^+ = 376), RHAL (MH^+ = 378), HTPNO (MH^+ = 374), and HNO (MH^+ = 392). CE capillary 100 cm × 50 μm I.D., run buffer 50 m*M* NH_4OAc, 10% methanol, 1% acetic acid (pH 4.1). Separation voltage 15 kV. ESI sheath liquid isopropanol–water–acetic acid (60:40:1, v/v/v), flow-rate 3 μl/min. Capillary–tube–skimmer voltages 39:114:5000 V, scan range of 450–125 a.m.u. at 3 s/decade, detection with a PATRIC focal plane detector.

MD, USA). Cytochrome P_{450} enzymes were induced by pretreatment with phenobarbitone (80 mg/kg/day × 3 days) prior to sacrifice of the mice on the fourth day [29].

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 (10 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 of original tissue. In control incubates, 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 nitrogen [30]. The residues were reconstituted in methanol (200 μl) and subjected to CZE separation with MS detection.

RESULTS AND DISCUSSION

Analysis of synthetic standards of putative HAL metabolites

Initial CZE–MS studies were performed on a mixture of HAL and six synthetic standards of previously demonstrated metabolites [6], namely CPHP, HP^+, HTP, HTPNO, RHAL and HNO (see Fig. 1 for structures). A CZE run buffer of 50 m*M* NH_4OAc containing 10% methanol and 1% acetic acid in water (pH 4.1) was used as previously described [12,13] to baseline resolve all seven structurally similar compounds (see the total ion-current electropherogram shown in Fig. 2). The limits of detection of each of these compo-

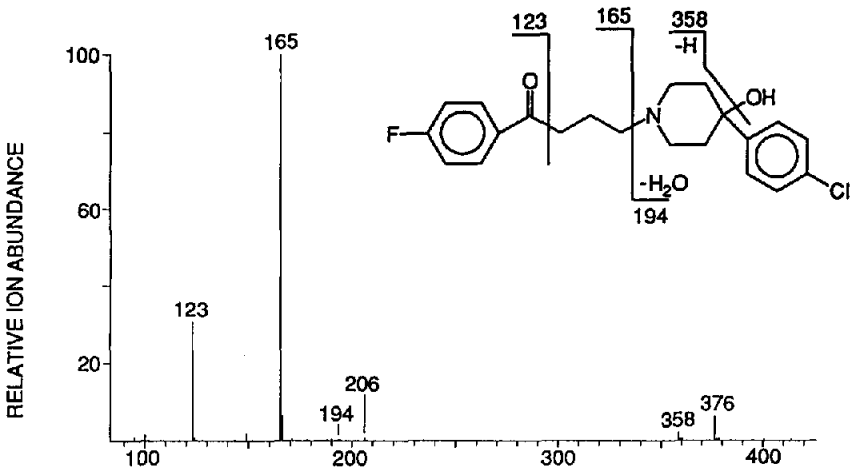

Fig. 3. An on-line CE–skimmer CID–MS spectrum of HAL. Capillary–tune–skimmer voltages 100:212:5000 V, scan range 400–50 a.m.u. at 2 s/decade. Detection with a PATRIC focal plane detector.

nents using the scanning PATRIC focal plane detector was ~100 fmol.

In further experiments, FBPA (see Fig. 1 for structure) was added to the standard mixture, since in previous studies [6,12] we had tentatively identified this compound as a component of microsomal incubates. It was, however, found that whilst this compound could be detected, its limit of detection was poor and no better than the previously reported 200 pmol by electron impact ionization [6].

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 as previously described by Smith *et al.* [31], lead to excitation of molecules by low energy collisions and can result in the production of structurally significant product ions.

An example of CZE–skimmer CID–MS is shown for the parent compound HAL in Fig. 3. 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 [6]. 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 I and Fig. 3. CZE–skimmer CID–MS product ion data for synthetic standards/putative metabolites of HAL are summarized in Table I. 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 [6]. 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.

It should be noted that the abundance of product ions produced in ESI–skimmer CID processes are variable and dependent predominately on the applied capillary and tube lens voltages. This is highlighted in Table II for the major product ions derived from HAL at *m/z* 165 and 123. As the capillary and tube lens voltages are increased, it is seen that the abundance of these ions relative to that of the precursor ion (MH^+ = 376) are increased. In addition, as these voltages are increased a product ion at *m/z* 95 corresponding to $[F–C_6H_5]^+$ is also observed. Such fragmentation

TABLE I

ON-LINE CE–SKIMMER CID–MS DATA FOR SYNTHETIC STANDARDS/PUTATIVE METABOLITES OF HAL[a]

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

[a] Note that the relative ion abundance data is highly dependent upon skimmer, capillary and tube lens voltage. This data was acquired at skimmer, capillary, and tube voltages of 5 kV, 100 V and 212 V, respectively.

pathways have been demonstrated in a previous study [32] to be useful for identifying modifications to aromatic ring systems.

Analysis of biologically derived HAL metabolite mixtures

Development of CZE–MS conditions to separate HAL metabolites was prompted by our interest in the metabolism of this widely used clinical drug. One area of particular interest is the difference in metabolism by humans and different animal species. Species dependent differences in metabolism can occur in both phase I and phase II stages and can be either qualitative and/or quantitative [33]. Such variations have been ascribed to differences in enzyme activity [34], and more recently, to molecular aspects of gene evolution [35]. Clearly, the problems raised by the variation in metabolism by different species for new and clinically used drugs is important in understanding the toxicological and pharmacological activity of metabolites.

TABLE II

ION ABUNDANCE DATA RECORDED FOR HAL FRAGMENT IONS IN SKIMMER CID EXPERIMENTS AT VARIOUS CAPILLARY AND TUBE LENS VOLTAGES

Capillary: tube voltages (V)	Relative ion abundance (%)			
	m/z 376	*m/z* 165	*m/z* 123	*m/z* 95
39 : 114	100	2	1	< 1
64 : 139	100	13	4	< 1
100 : 175	100	52	43	8
138 : 212	80	46	100	48
163 : 237	87	46	100	61

We investigated the *in vitro* metabolism of HAL by both mouse and guinea pig hepatic microsomes using the developed CZE–MS conditions. The ion electropherograms resulting from the analysis of the microsomal incubates, after solid phase clean-up, are shown in Fig. 4 (mouse) and Fig. 5 (guinea pig). Inspection of the data recorded for the mouse hepatic microsomal incubate revealed prominent ions at *m/z* 212, 354, 358, 376 and 392 corresponding to CPHP, HP^+, HTP, unmetabolised HAL, and HNO, respectively. Under the CZE conditions used, biologically derived RHAL migrated with HAL, however, a prominent ion at *m/z* 378 corresponding to the chlorine isotope contribution of HAL plus the MH^+ of RHAL suggested its presence. The prominent ion at *m/z* 380 corresponding to the

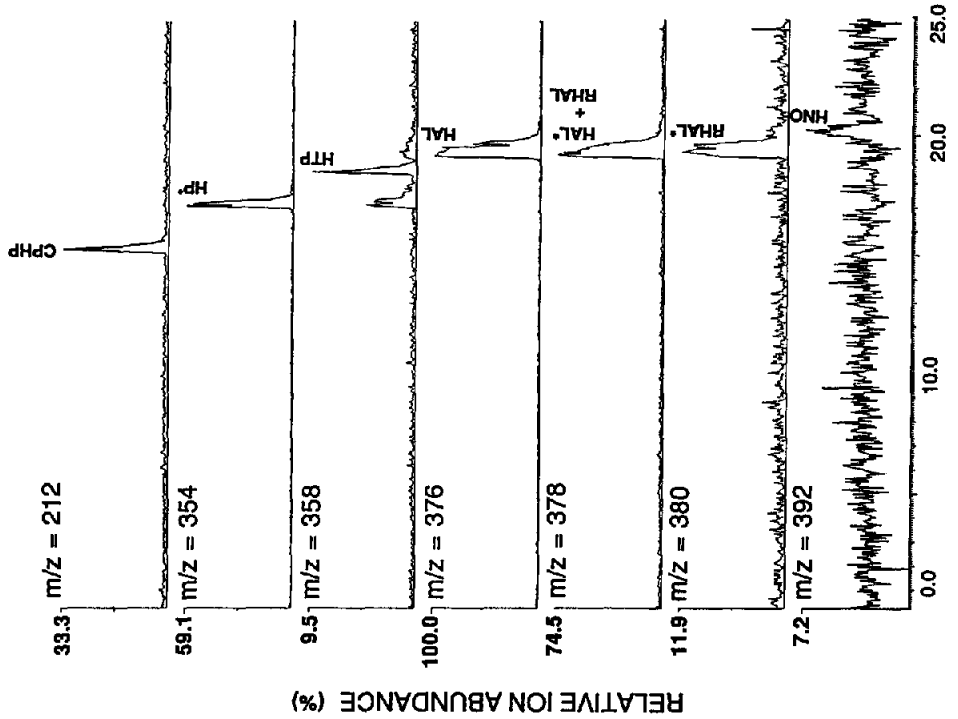

Fig. 4. Ion electropherograms of phase I HAL metabolites detected in an *in vitro* incubation with mouse hepatic microsomes. HAL* and RHAL* denote the ^{37}Cl isotope responses of these compounds. Note that relative ion abundance is based on peak-area measurements relative to *m/z* 376 of HAL. Conditions as for Fig. 2 except CE capillary dimensions were 65 cm × 50 μm I.D.

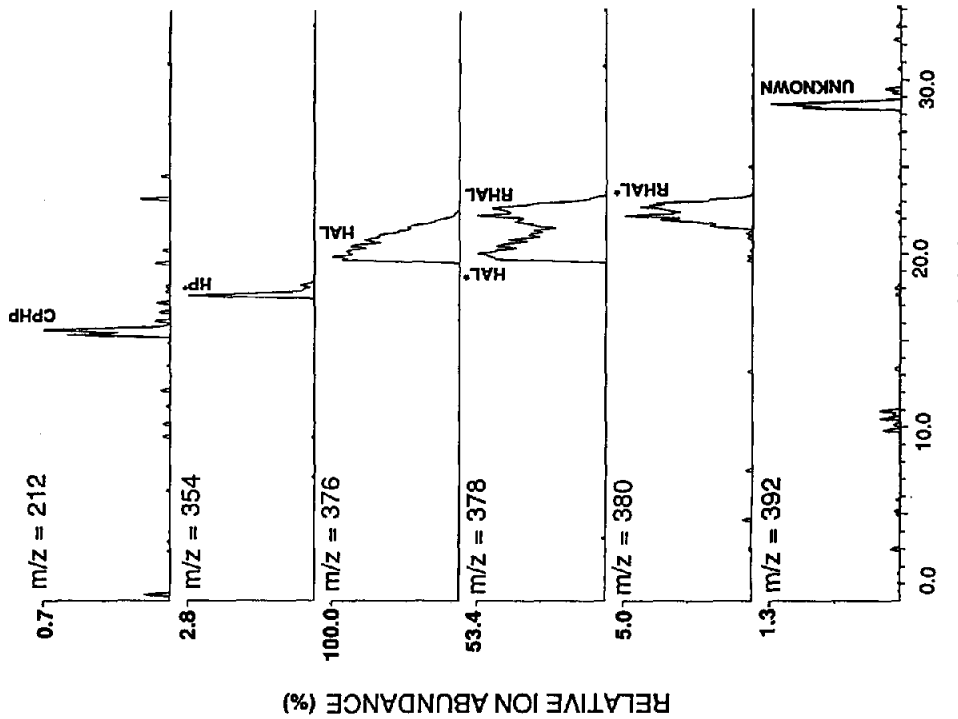

Fig. 5. Ion electropherograms of phase I HAL metabolites detected in an *in vitro* incubation with guinea pig hepatic microsomes. HAL* and RHAL* denote the ^{37}Cl isotope responses of these compounds. Note that relative ion abundance is based on peak-area measurements relative to *m/z* 376 of HAL. Conditions as for Fig. 4.

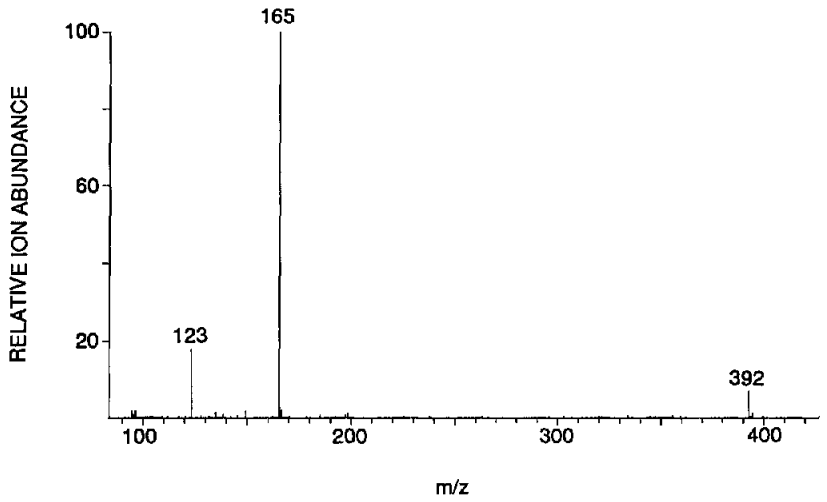

Fig. 6. An on-line CE–skimmer CID–MS spectrum of the unknown phase I HAL metabolite detected in the guinea pig microsomal incubate. Conditions as for Fig. 3.

chlorine isotope contribution 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 resolved in the synthetic standard mixture (Fig. 2) and the guinea pig microsomal incubate (Fig. 5). There was also tentative evidence that FBPA (MH^+ = 197) had also been produced in this incubate (data not shown).

In contrast, analysis of the guinea pig hepatic microsomal incubation mixture by CZE–MS revealed the presence of ions at *m/z* 212, 354, 376, and 378 corresponding to CPHP, HP^+, unmetabolised HAL and RHAL, respectively (Fig. 5). Again, there was tentative evidence that FBPA was a component of this incubation mixture (data not shown). The poor peak width and shape of HAL is due to overloading the capillary with unmetabolized drug (*m/z* 376, and *m/z* 378 — chlorine isotope contribution). However, at present, we cannot explain why the signal at *m/z* 380 corresponding to the chlorine isotope ion of RHAL should also be so poor, since the amount of metabolite present has been shown to be significantly less than HAL [12].

Further investigation of the guinea pig sample revealed an ion at *m/z* 392, suggesting the presence of HNO. However, the relative (to HAL) migration time was longer than that of authentic standard HNO. Subsequently, a guinea pig microsomal incubation was spiked with authentic HNO standard, whereupon CE analysis revealed two distinct responses corresponding to HNO and the unknown compound [12].

All ions detected by CZE–MS in both the mouse and guinea pig microsomal samples were subsequently subjected to CZE–skimmer CID–MS. Skimmer CID voltage conditions were similar to those employed for standard compounds shown in Table I (*i.e.* capillary–tube–skimmer, 100:212:5000 V) in order to maximise fragmentation but still retain an abundant precursor ion (see Table II). Fragment ions derived from precursor ions at *m/z* 212, 354, 358, 376, and 392 (mouse hepatic microsomal incubation) were identical (results not shown) to fragment ions derived from synthetic standards CPHP, HP^+, HTP, HAL and HNO respectively (see Table I). Skimmer CID spectra of ions present in the guinea pig sample confirmed the presence of CPHP, HP^+, and RHAL.

A CZE–skimmer CID–MS spectrum of the compound of *m/z* 392 found in the guinea pig microsomal incubation is shown in Fig. 6. Since the major ion of this spectrum is *m/z* 165, which corresponds to $[F–C_6H_4–C(O)CH_2CH_2CH_2]^+$,

it is apparent that the metabolic modification of HAL had occurred at the piperidine end of the molecule. Hence, this compound is apparently isomeric with HNO. However, the lack of fragmentation with charge retention on the substituted piperidine prevented full structural elucidation of this compound. Indeed, the skimmer CID spectrum of this compound was similar to that of standard HNO (see Table I). We are continuing our studies of this compound by employing complimentary physical methods, *e.g.* NMR, so as to fully elucidate the structure of this metabolite of the guinea pig microsomal incubation of HAL.

CONCLUSIONS

Our study has shown that both CE–MS and CE–skimmer CID–MS techniques enable rapid detection and metabolite identification in drug metabolism studies. Furthermore, we have demonstrated that such methodology can be used to investigate the structural characteristics of novel metabolites. Specifically, we have investigated the *in vitro* metabolism of HAL and have shown, by CE–MS and CE–skimmer CID–MS, a clear difference in metabolism exhibited by mouse and guinea pig in both a qualitative and quantitative manner (see ion abundance values on Figs. 4 and 5). The induced mouse microsomes also metabolised HAL more efficiently than the guinea pig microsomes. However, the guinea pig microsomal incubate contains an unknown metabolite (m/z 392) that was not detected in the mouse hepatic microsomal incubate of HAL.

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