ApplicationNOTE

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CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRIC AND TANDEM MASS SPECTROMETRIC ANALYSIS AND IDENTIFICATION OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND THEIR METABOLITES IN HUMAN URINE *

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Abstract

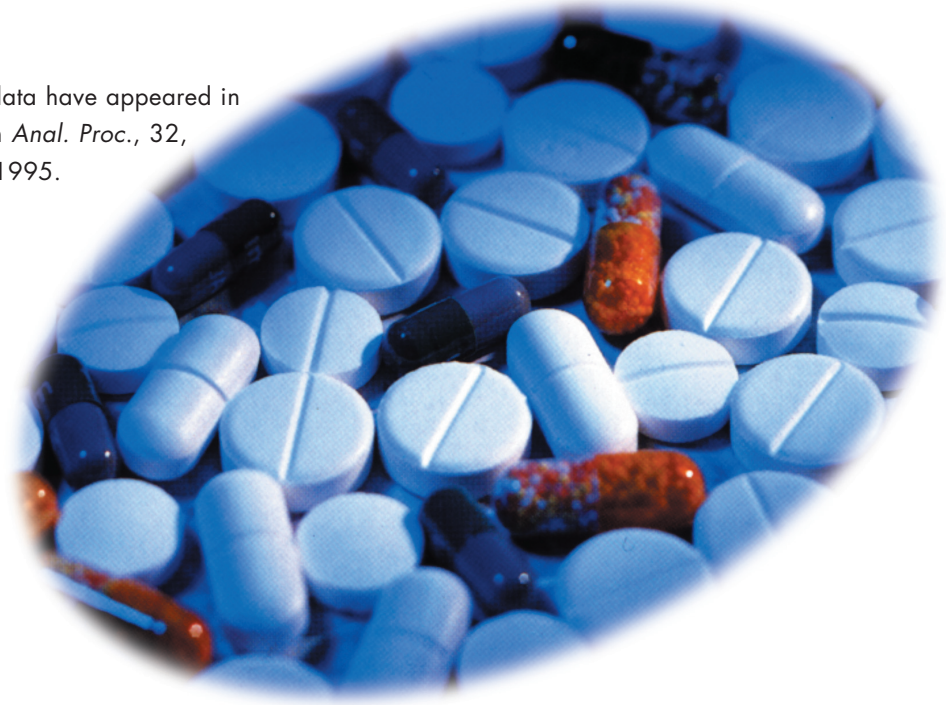
Capillary electrophoresis (CE) was coupled via a triaxial sheath flow interface directly to the negative ionization electrospray (ES) atmospheric pressure source of a commercially available tandem quadrupole mass spectrometer (MS). The CE eluent was supplemented by a solvent make-up flow and this, together with a low flow nebulizer gas, produced a reliable and robust CE-ES-MS interface.

Human urine extracts, after independent administration of three non-steroidal anti-inflammatory drugs, were analyzed and the major metabolites identified using a combination of molecular weight (i.e. $(M-H)^-$) and fragmentation data generated from both in-source collision induced dissociation and low energy tandem MS (MS-MS). The in-source collision induced dissociation and MS-MS product ion scanning spectra provided similar information.

MS-MS precursor ion scanning was used for the ibuprofen-containing urine extract to screen specifically for glucuronide metabolites, all of which produced a common fragment ion at *m/z* 175 in negative ionization electrospray.

In conclusion, on-line CE-ES-MS (-MS) proved to be a practical, viable technique for metabolite monitoring and identification of the drugs described, while minimizing sample clean-up.

* These data have appeared in part in *Anal. Proc.*, 32, 459, 1995.



MICROMASS MS TECHNOLOGIES

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Introduction

Capillary electrophoresis (CE) and related techniques such as micellar electrophoresis have found increasing use in the area of drug and metabolite analysis in biological fluids[1] due to the low sample and solvent consumption and high separation efficiencies. In parallel, CE coupled with electrospray (ES) - mass spectrometry (MS) is emerging as a viable on-line technique for a variety of routine analyses[2,3], and has been applied to drug analysis[4,5]. In this paper, the high resolution of CE combined with the sensitivity and specificity of ES-MS and tandem ES-MS has been used to produce a practical method for the detection and identification of three non-steroidal anti-inflammatory drugs and their metabolites in solid phase extracts of human urine.

The drugs chosen were ibuprofen, flurbiprofen and aspirin, (Schematic 1), all widely administered and all of which produce highly polar metabolites amenable to negative ionization ES. The aim of the analyses was to produce both molecular mass information and to generate structurally useful fragment ions to aid the identification of the metabolites. By studying known drugs the validity of this approach can be estimated, and if successful the methodology then applied to more novel drug metabolism projects.

Experimental

All solvents were of HPLC grade purity, and all reagents and chemicals of analytical grade purity. All were used without further purification.

Drug Administration and Sample Preparation

Urine was obtained from a healthy male volunteer for the period 0 to 2 hr post-administration of a standard therapeutic dose of one of the chosen drugs: ibuprofen (400 mg), flurbiprofen (100 mg), or aspirin (600 mg), and then stored at -20°C until required for extraction.

Solid phase extraction (SPE) of the urine was performed using C_{18} bonded SPE cartridges containing 500 mg of sorbent (Bond Elut, Varian, supplied by Jones Chromatography Ltd., Hengoed, UK). The cartridges were activated prior to extraction by washing with methanol (5 mL) followed by 0.1 M HCl (5 mL). Samples of urine (2 mL) were acidified to pH 2 with 0.1M HCl and then applied to the activated cartridges. The cartridges were then washed with 0.1M HCl (1 mL) followed by elution with methanol (5 mL). The collected methanolic eluates were reduced to dryness using a stream of nitrogen, and stored at -20°C until required for analysis. For CE-MS the dry residues were reconstituted in methanol (200 (µL).

Capillary Electrophoresis

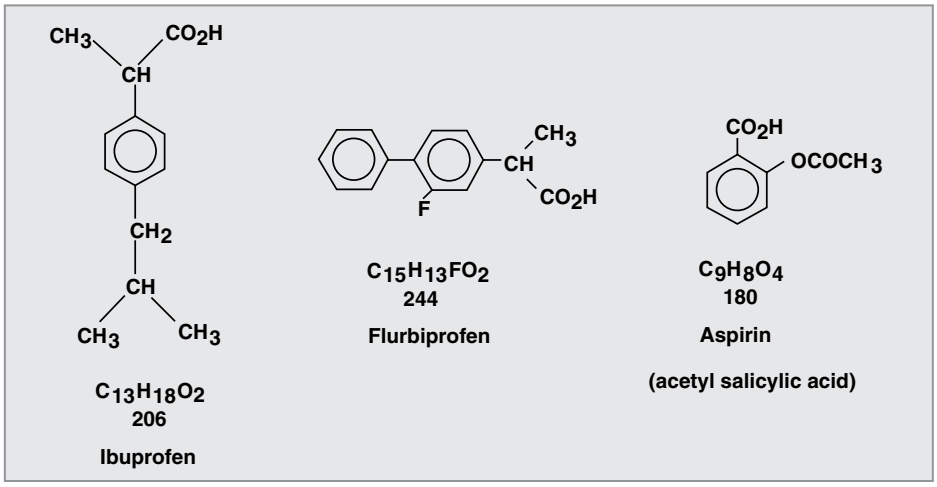
CE was performed with an uncoated fused silica capillary (75 µm i.d. x 375 µm o.d. x 100 cm length) using a Beckman P/ACE System 2100 (Beckman Instruments Inc., Palo Alto, USA) with in-line UV detection (200 nm) 19 cm from the injection end of the capillary. New CE capillaries were flushed with 1M NaOH, then water and finally the buffer in use for the analyses. In-between subsequent analyses the CE capillary was flushed with buffer only. A pressure injection (5 or 10 seconds) representing approximately 30 nL was used to introduce the sample into the capillary and then a voltage (20 kV) applied to effect separation. The CE buffer used was 20 mM aqueous ammonium acetate taken to pH 9 with aqueous ammonia solution.

Mass Spectrometry

The CE capillary was taken directly into the atmospheric pressure ES source via the CE-ES triaxial sheath flow probe/interface of a Micromass® Quattro II™ (Waters Corporation, Altrincham, Cheshire, UK) tandem quadrupole mass spectrometer. The low flow of liquid emerging from the CE capillary necessitated the use of a make-up flow of water: propan-2-ol (20:80, v:v, 8 (µL/minute) which was introduced coaxially through the probe thus ensuring that mixing of the two mobile phases took place at the probe tip inside the source and hence electrophoretic resolution was undisturbed.

The probe tip was held at -4 kV and nitrogen was used as both the drying gas and nebulizing gas, the latter being introduced through the triaxial probe. The source temperature was maintained at 70°C. Negative ionization was used for all the analyses and mass accuracy was ensured by calibration on a separate introduction of a sugar mixture using negative ionization ES analysis. All analyses were acquired at unit resolution (50% valley definition).

Molecular mass information (i.e. $(M-H)^-$ ions) was obtained using a low sampling cone voltage (30 V). In order to generate fragment ions in the source, this value was increased to 50 V. For tandem mass spectrometry (MS-MS) analyses, argon gas was introduced into the collision cell, which is situated between the first and second quadrupole analyzers, and a collision energy of 15 - 20 eV set.



Schematic 1. Chemical structures and formulae of ibuprofen, flurbiprofen and aspirin.

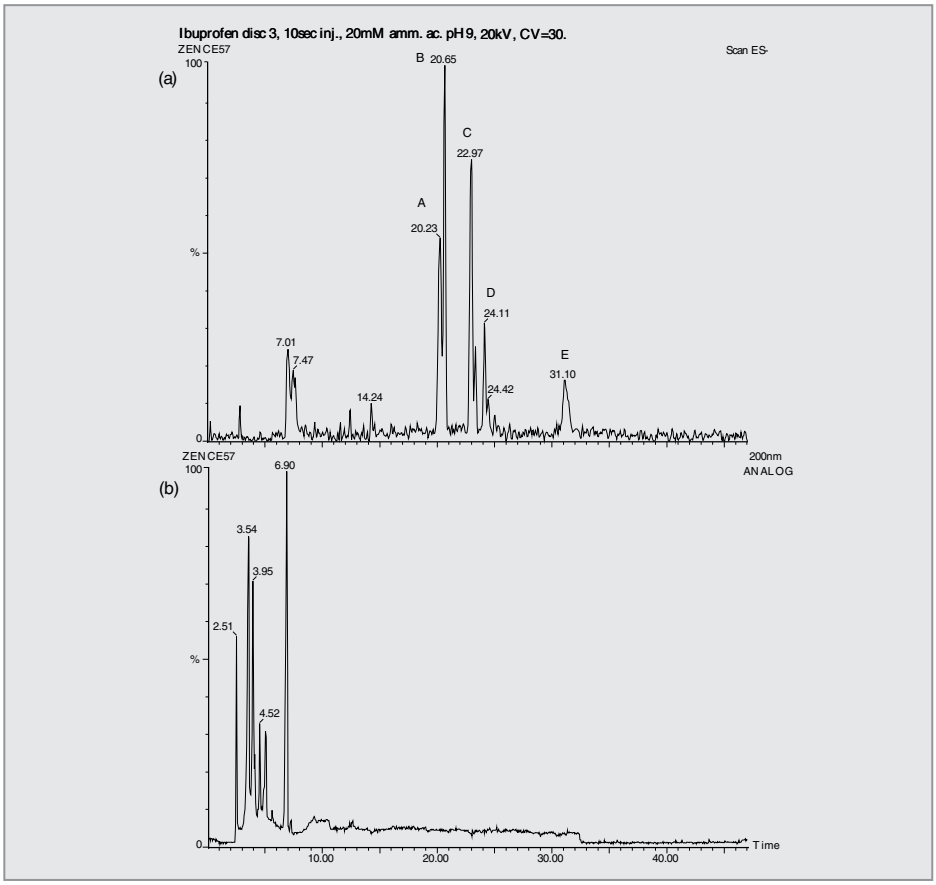


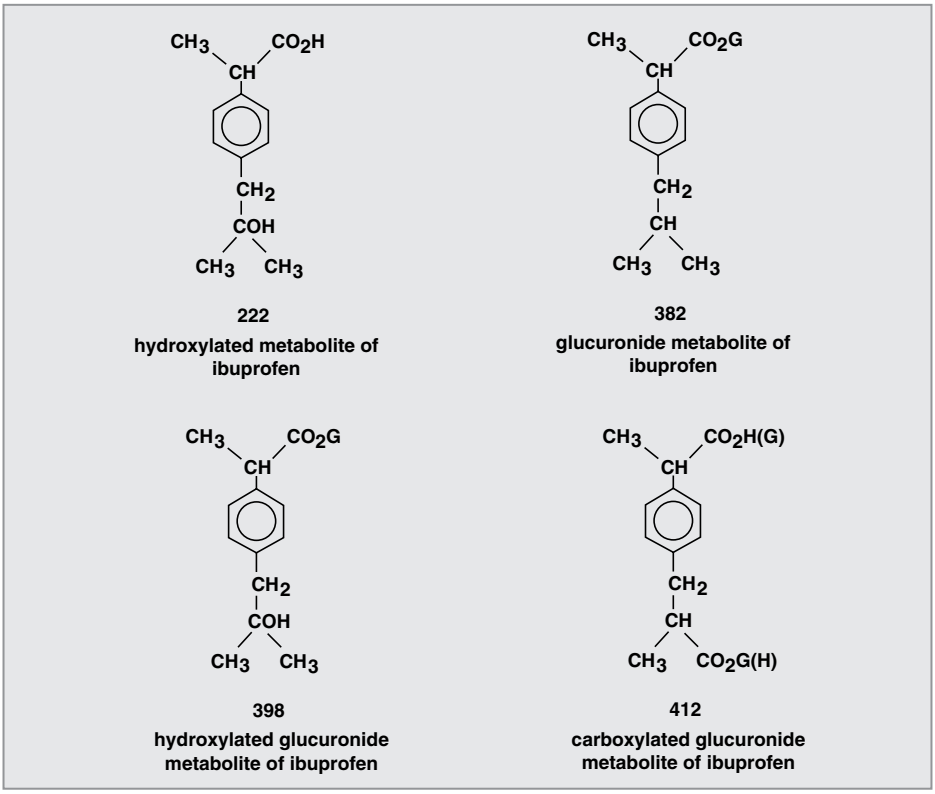
Figure 1. (a) CE-MS total ion electropherogram for the separation of ibuprofen metabolites isolated from human urine by solid phase extraction. The order of elution of the peaks was: A. hydroxy ibuprofen glucuronide, 20.23 min.; B. ibuprofen glucuronide, 20.65 min.; C. hydroxy ibuprofen, 22.97 min.; D. ibuprofen, 24.11 min.; E. carboxylated ibuprofen glucuronide, 31.10 min. (b) in-line CE-UV (200 nm) electropherogram.

Results and Discussion

Ibuprofen

Following oral administration to humans, ibuprofen is rapidly excreted[6] as a mixture including the drug and its hydroxy and carboxylic acid metabolites, together with the glucuronide conjugates of these three species. Ibuprofen and its metabolites are efficiently extracted by SPE onto C_{18} bonded silica which renders this a rapid, simple and convenient clean-up procedure from urine.

The CE-ES-MS analysis of the ibuprofen urine extract produced the UV and total ion mass spectrometric electropherograms shown in Figure 1. The difference in retention times between the two traces results from the fact that the UV detection is taking place approximately one-fifth of the way along the separation column. A low sampling cone voltage (30V) had been set in the ionization source for this analysis to optimize the production of the $(M\text{-}H)^-$ ions, and from these ions the molecular masses of the components contained in this extract were established. The major components detected had molecular masses consistent with those expected for ibuprofen (MW 206), its side-chain hydroxylated metabolite (MW 222), the glucuronides of both these species (MWs 382 and 398 respectively), and the glucuronide of the side-chain oxidized carboxylic acid metabolite (MW 412), (Schematic 2).



Schematic 2. Chemical structures of the major ibuprofen metabolites.

Figure 2 shows the electropherograms of the $(M\text{-}H)^-$ ions of these components, in comparison with the total ion electropherogram. The CE-ES-MS peak width measured from the electropherogram is typically 12 seconds for these compounds.

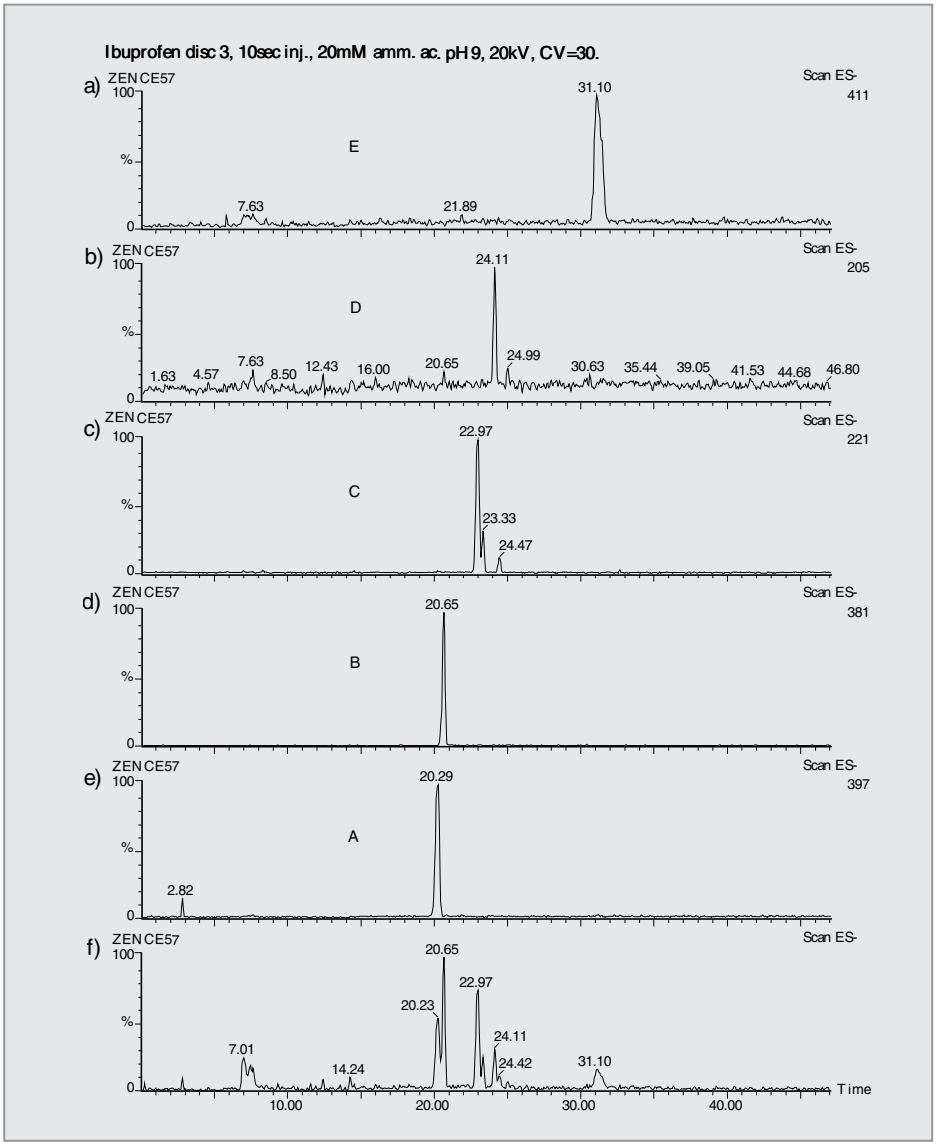


Figure 2. CE-MS total ion (f) and selected ion electropherograms, (a) - (e), for the separation of ibuprofen metabolites isolated from human urine by solid phase extraction, showing the $(M\text{-}H)^-$ ions of ibuprofen, m/z 205; hydroxy ibuprofen, m/z 221; ibuprofen glucuronide, m/z 381; hydroxy ibuprofen glucuronide, m/z 397; carboxylated ibuprofen glucuronide, m/z 411.

The analysis was repeated using a higher sampling cone voltage (50V) in order to achieve in-source collision induced dissociation and generate structurally informative fragment ions for all of the components in the urine extract. The low and high cone voltage spectra obtained for the glucuronide conjugates of ibuprofen, hydroxylated ibuprofen, and carboxylated ibuprofen are compared in Figure 3. As these spectra show, in-source collision induced dissociation generates structurally useful information by producing diagnostic fragment ions. For example, the three glucuronide conjugates all exhibit a fragment ion at *m/z* 175 resulting from cleavage of the glucuronic acid portion of the molecules, and all three show a complementary loss of 176 amu from their $(M-H)^-$ ions to form the $(M-H)^-$ ions of the corresponding aglycones (i.e. *m/z* 205, 221, and 235) and/or decarboxylated aglycones (i.e. *m/z* 161, 177, and 191) of ibuprofen, hydroxylated ibuprofen and carboxylated ibuprofen respectively. It can also be observed that the intensity of the sodium adduct ions $(M+Na-H)^-$, compared to the $(M-H)^-$ ions, is greater when a higher sampling cone voltage is used, implying that the sodiated adduct is the more stable of the two. These results indicate the potential of in-source collision induced dissociation on a single quadrupole mass spectrometer to produce structurally informative fragment ions.

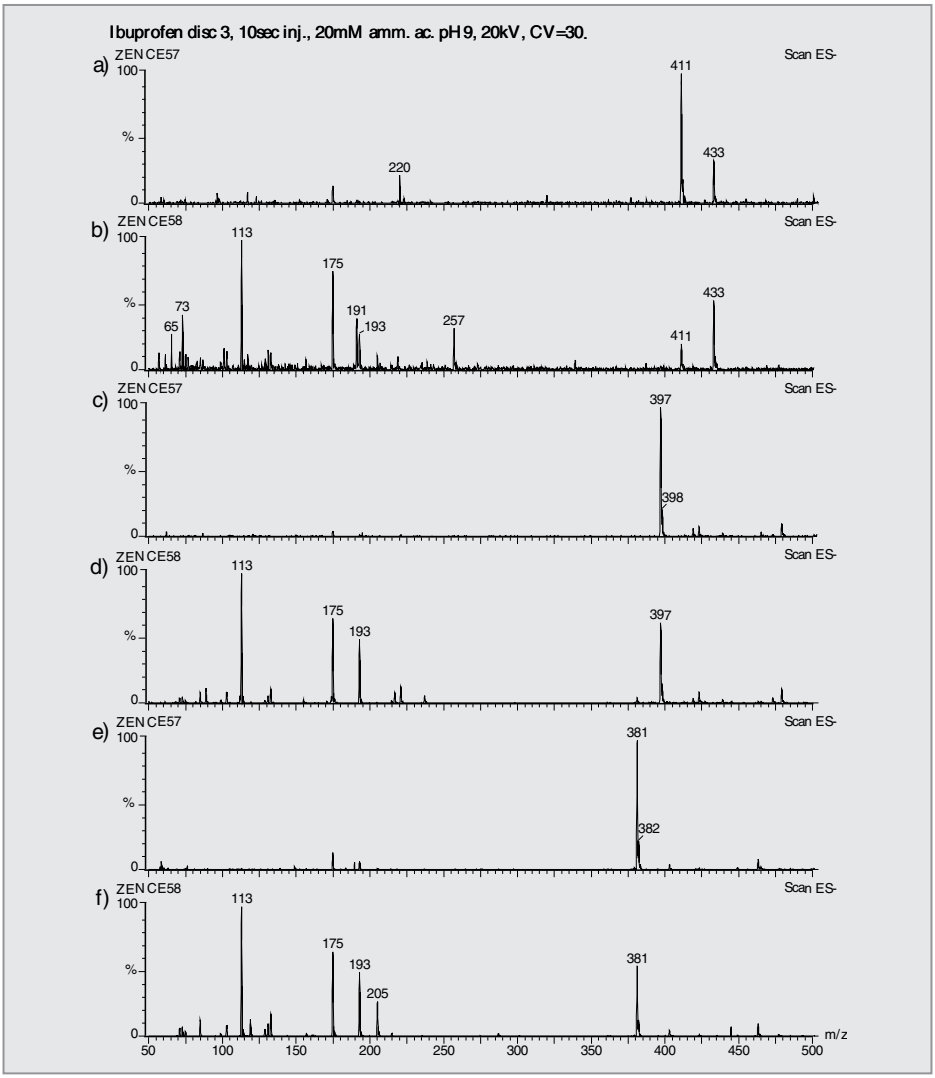


Figure 3. The low and high sampling cone voltage spectra of ibuprofen glucuronide (e and f), hydroxy ibuprofen glucuronide (c and d), and carboxylated ibuprofen glucuronide (a and b).

For more specific fragmentation, and hence structural information, MS-MS can be used with on-line CE-ES. Figure 4 shows the MS-MS product ion spectra obtained from selectively and independently transmitting the $(M\text{-}H)^-$ ions of the same three glucuronide conjugates through the first quadrupole analyzer into the collision cell, and then monitoring the fragment ions arising directly from these using the second quadrupole analyzer. Many similarities between the product ion MS-MS spectra and the in-source collision induced dissociation spectra are apparent, in particular the characteristic glucuronide ions described for Figure 3. MS-MS data have the advantage of not exhibiting solvent-related ions and contain only those fragment ions which arise directly from the selected precursor.

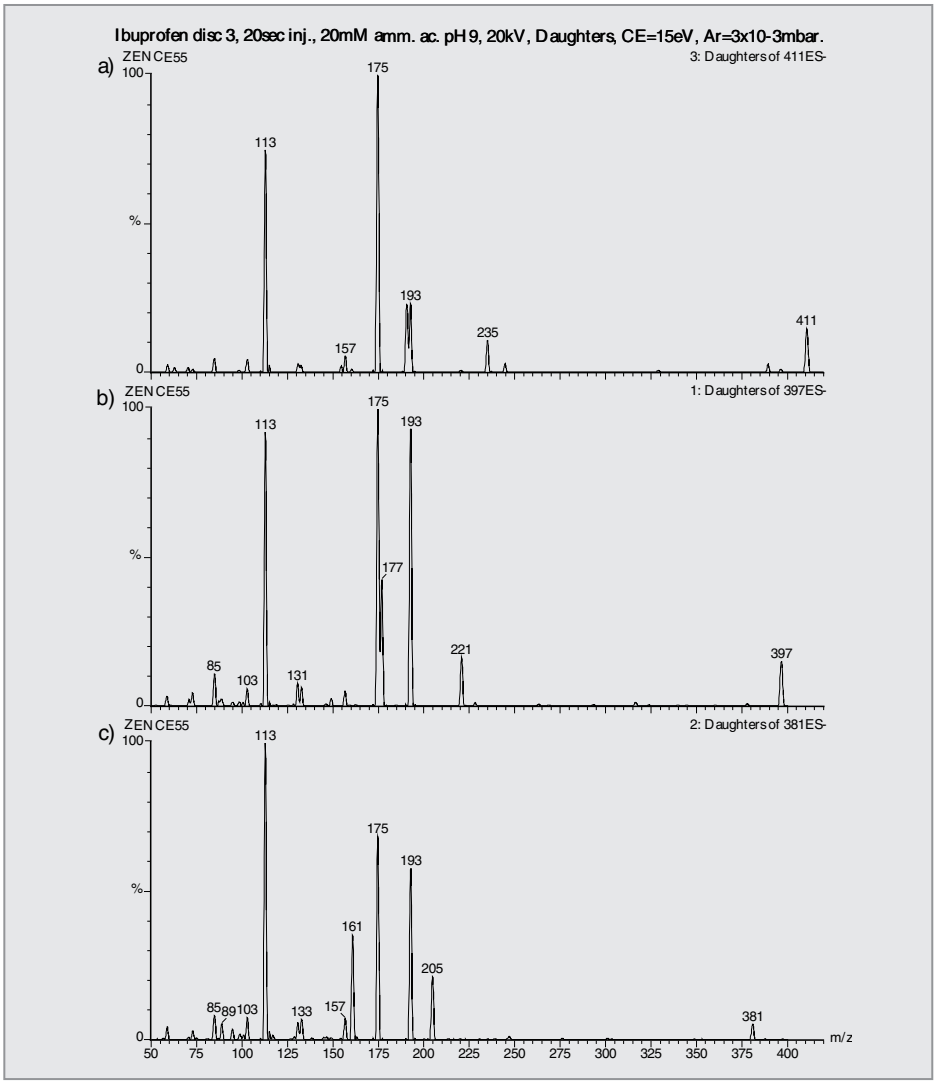


Figure 4. CE-MS-MS product ion spectra of the ibuprofen glucuronide conjugates:
(a) carboxylated ibuprofen glucuronide;
(b) hydroxy ibuprofen glucuronide;
(c) ibuprofen glucuronide.

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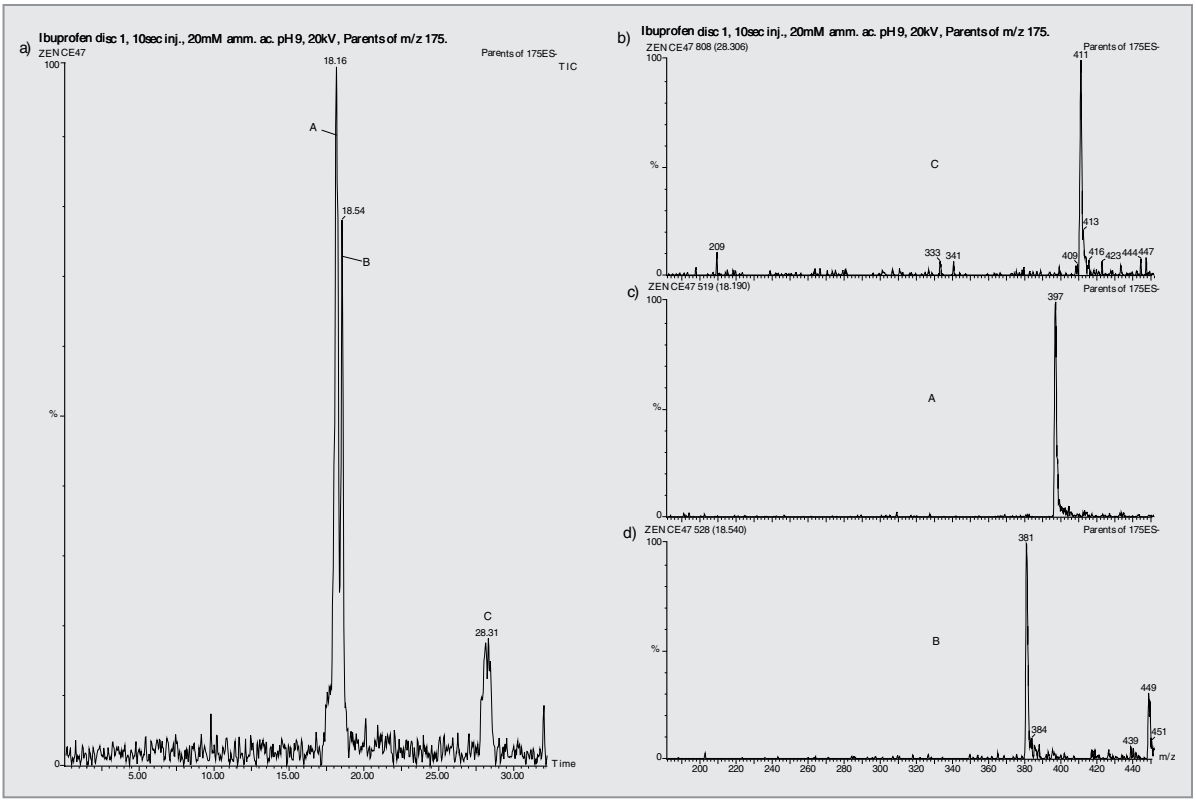

Figure 5. CE-MS-MS ibuprofen metabolite precursor ion data from m/z 175:

(a) total ion electropherogram monitoring the precursor ions from m/z 175 in order to detect the glucuronide conjugates, showing A. hydroxy ibuprofen glucuronide; B. ibuprofen glucuronide; C. carboxylated ibuprofen glucuronide;

(b) The MS-MS precursor ion spectrum for carboxylated ibuprofen glucuronide;

(c) The MS-MS precursor ion spectrum for hydroxy ibuprofen glucuronide;

(d) The MS-MS precursor ion spectrum for ibuprofen glucuronide.

Ester glucuronide conjugates are very common metabolites of carboxylate-containing drugs and the ready fragmentation of these metabolites to generate a common ion at *m/z* 175 can be exploited using MS-MS to detect and identify selectively these conjugates in complex media such as urine extracts. This approach is illustrated in Figure 5 which shows the electropherogram from the precursor ion scan of *m/z* 175. In this case the second quadrupole analyzer was set to transmit only the *m/z* 175 ion while the first quadrupole analyzer was scanned routinely, with the result that only those components which produce a fragment at *m/z* 175 are monitored. The electropherogram shows three distinct components, whose precursor ion spectra show specifically those ions from which the *m/z* 175 fragment arises directly. The data indicate that the three components selectively monitored are indeed the three glucuronide conjugates discussed, and this screening procedure provides a rapid and specific means of identifying a particular set or type of compound in a complex extract.

Flurbiprofen

Flurbiprofen is another non-steroidal anti-inflammatory with some structural similarities to ibuprofen. In humans, after oral administration, it is also subject to both "phase 1" metabolic hydroxylation reactions and also "phase 2" reactions to form glucuronide conjugates. After solid phase extraction from human urine, on-line CE-ES-MS was used to separate and identify the components in the extract. Figure 6 shows the total ion mass spectrometric electropherogram from the CE-ES-MS analysis of the flurbiprofen urine extract using a low sampling cone voltage to optimize the $(M\text{-}H)^-$ ions. The peaks corresponding to flurbiprofen (MW 244), flurbiprofen glucuronide (MW 420) and the glucuronide of the hydroxylated metabolite of flurbiprofen (MW 436) have been identified on the electropherogram, with retention times of 20.60, 17.56 and 17.34 minutes respectively.

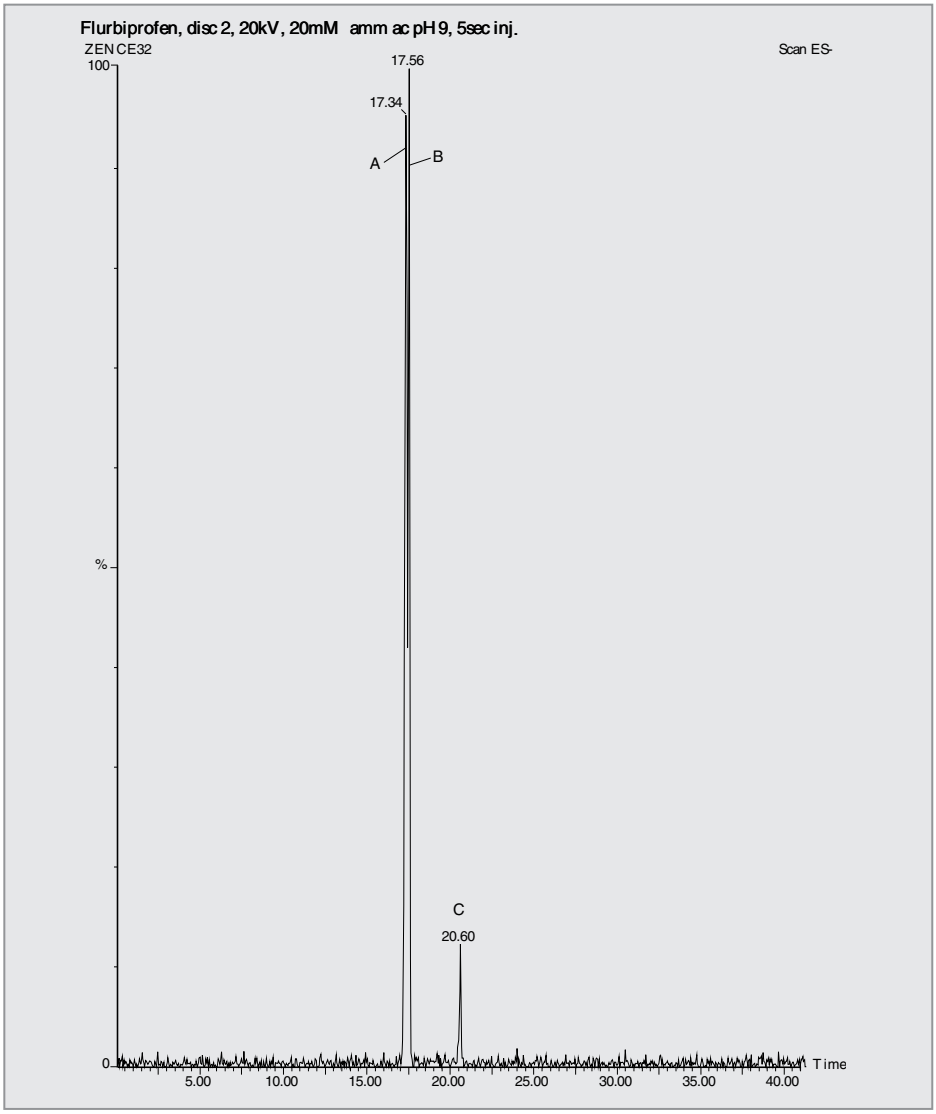

Figure 6. CE-MS total ion electropherogram for the separation of flurbiprofen metabolites isolated from human urine by solid phase extraction. The order of elution of the peaks was: A. hydroxy flurbiprofen glucuronide, 17.34 min.; B. flurbiprofen glucuronide, 17.56 min.; C. flurbiprofen, 20.60 min.

The analysis was also performed with a high sampling cone voltage to induce in-source fragmentation, and complementary pairs of spectra for the two glucuronide conjugates are presented in Figure 7. As the spectra indicate, the $(M\text{-}H)^-$ ions are easily distinguished together with diagnostic fragment ions at *m/z* 175 for the glucuronic acid moieties, and their corresponding decarboxylated aglycone ions. Although the two glucuronides were barely resolved on the CE-MS electropherogram, clean spectra without interfering ions from other components have been obtained for both components.

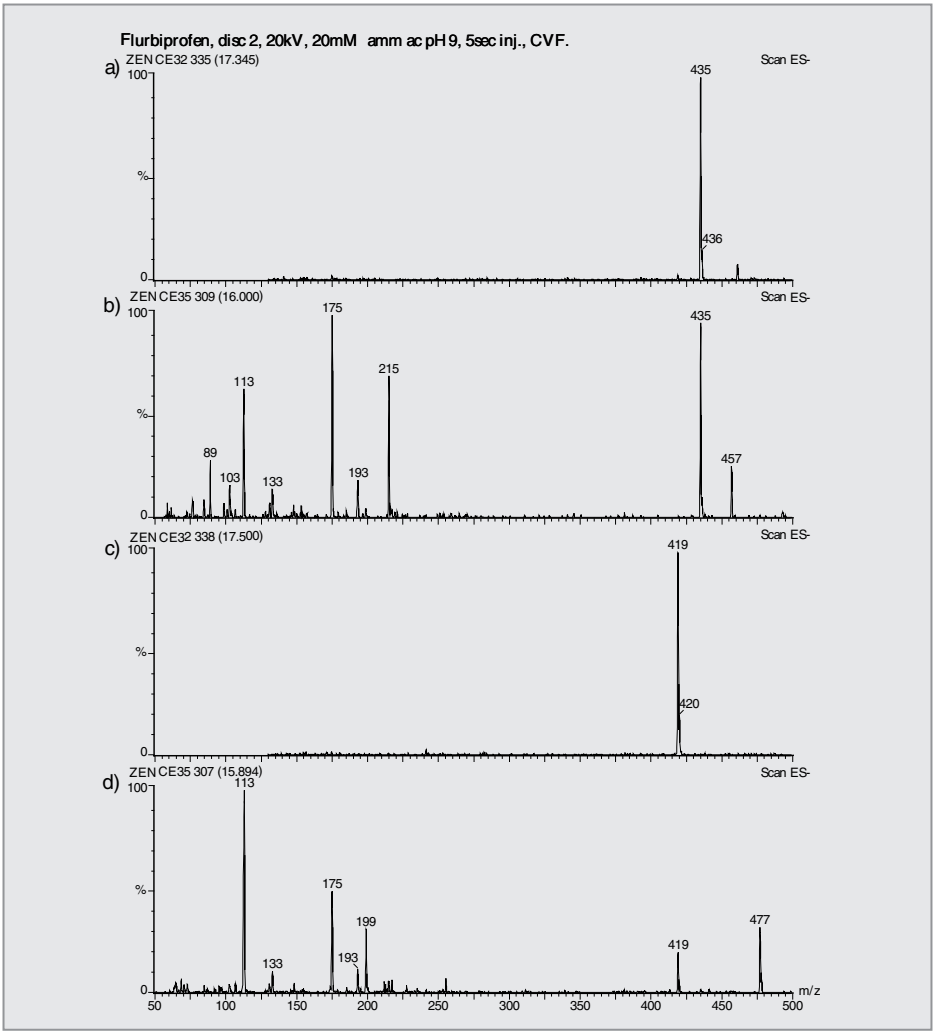

Figure 7. The low and high sampling cone voltage spectra of hydroxy flurbiprofen glucuronide (a and b), and flurbiprofen glucuronide (c and d).

Aspirin

Aspirin (acetylsalicylic acid)[7] is readily metabolized in humans to salicylic acid (2-hydroxybenzoic acid), which is then mostly conjugated to glycine and excreted in urine as salicylhippuric acid. The glucuronide metabolites (both ester and ether) of salicylic acid are also produced to a lesser extent, and in addition minor metabolites include the hydroxylated metabolite gentisic acid, its sulphate and glucuronic acid conjugate, and gentisuric acid (the glycine conjugate).

The CE-ES-MS total ion electropherogram from the analysis with in-line UV detection using a low sampling cone voltage to optimize the $(M\text{-}H)^-$ ions is illustrated in Figure 8. The peaks were assigned as follows: hippuric acid (MW 179; 22.20 minutes), salicylhippuric acid (MW 195; 28.40 minutes), and salicylic acid (MW 138; 29.90 minutes).

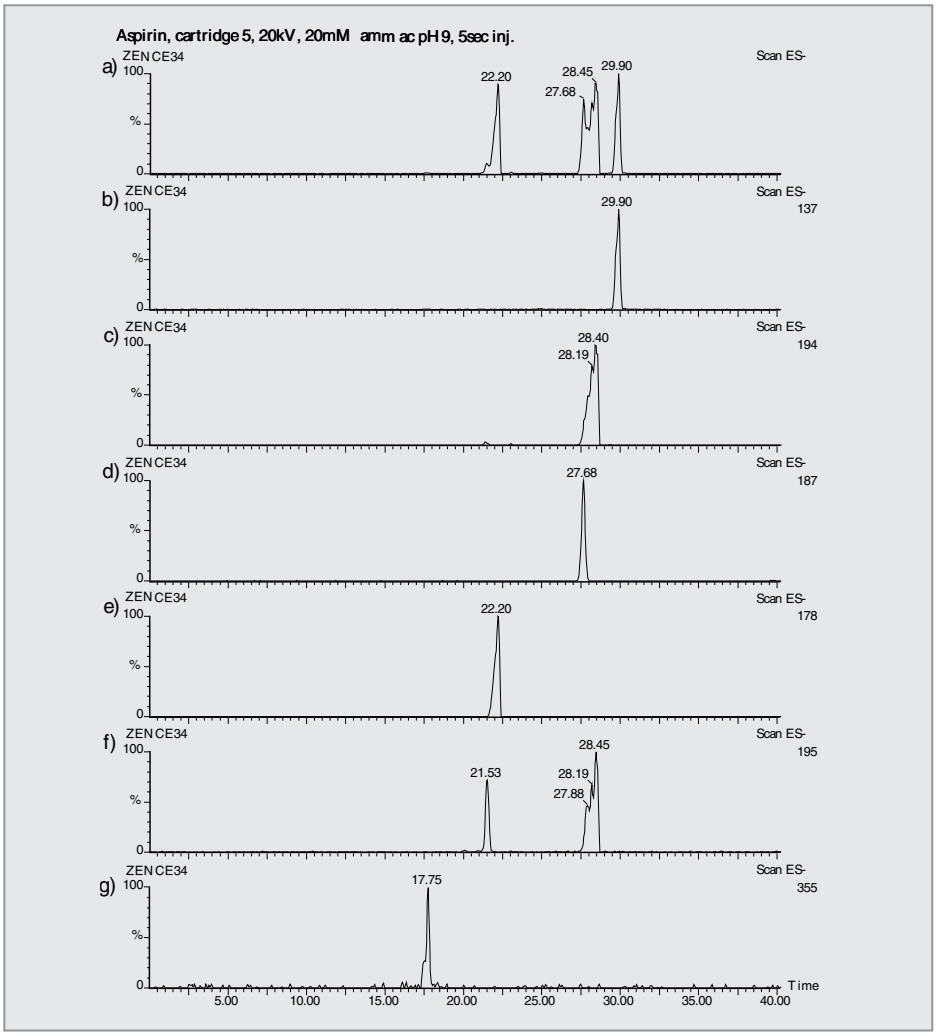

Figure 8. (a) CE-MS total ion and (b) - (g) selected ion electropherograms for the separation of aspirin metabolites isolated from human urine by solid phase extraction.

The analysis was repeated at a high sampling cone voltage and also using on-line MS-MS experiments in order to generate structural information. For example, Figure 9 compares the low sampling cone voltage spectrum for the component of retention time 28.40 minutes (Figure 8) with the MS-MS product ion spectrum monitoring fragments of *m/z* 194. The assignment of salicylhippuric acid was made by observation of the $(M\text{-}H)^-$ ion at *m/z* 194 in the former spectrum and the diagnostic fragment ions at *m/z* 150 and 93 in the latter spectrum.

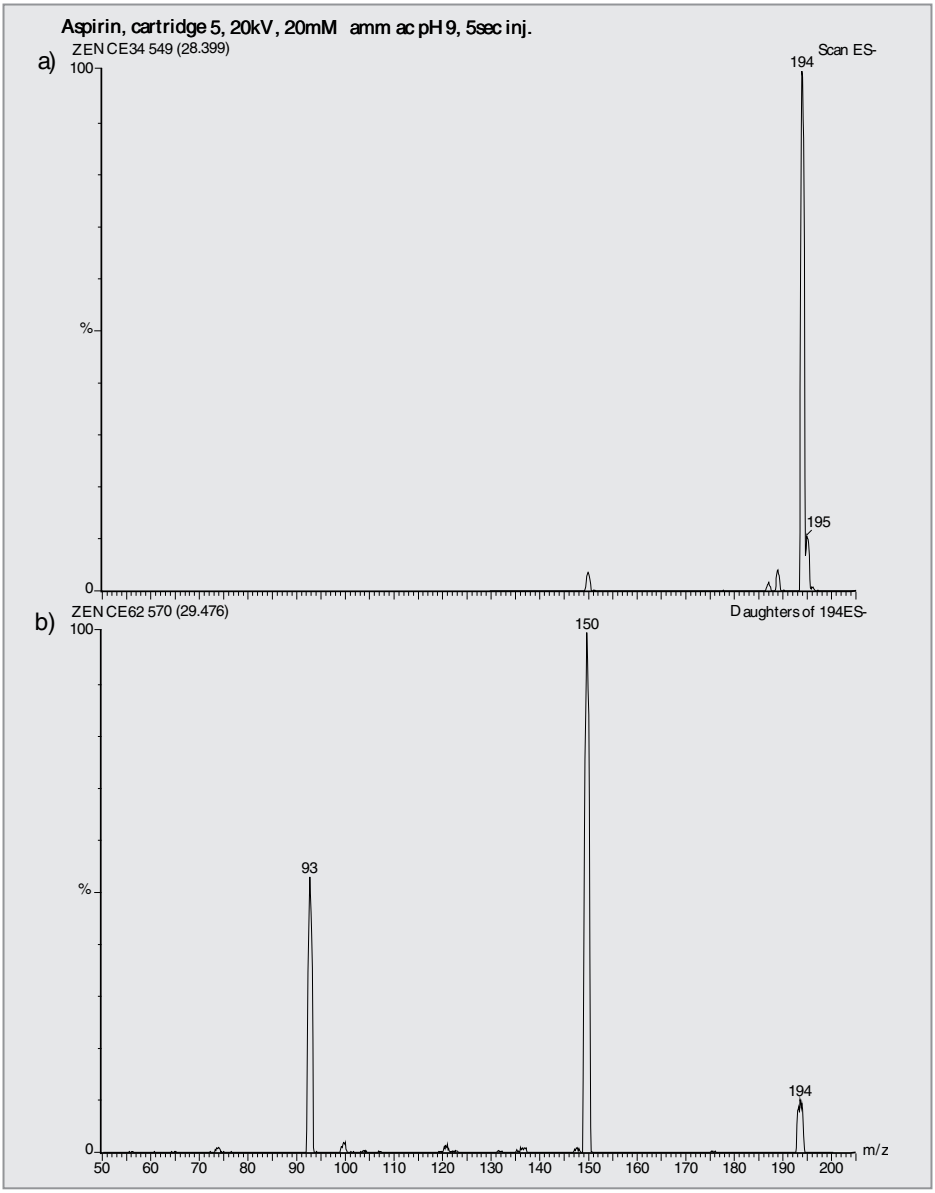

Figure 9. (a) The low sampling cone voltage spectrum of salicylhippuric acid compared with (b) its MS-MS product ion spectrum monitoring fragment ions from m/z 194.

A Constant Neutral Loss (CNL) CE-ES-MS-MS experiment monitoring those ions which lose 44 amu was performed on this urine extract to highlight specifically those components containing carboxylate functional groups by scanning both quadrupole analyzers with an offset of 44 amu. Figure 10 shows two components of the mixture monitored by this method: salicylhippuric acid (MW 195) and hippuric acid (MW 179), both glycine conjugates. The method could constitute a rapid screening technique for complex extracts.

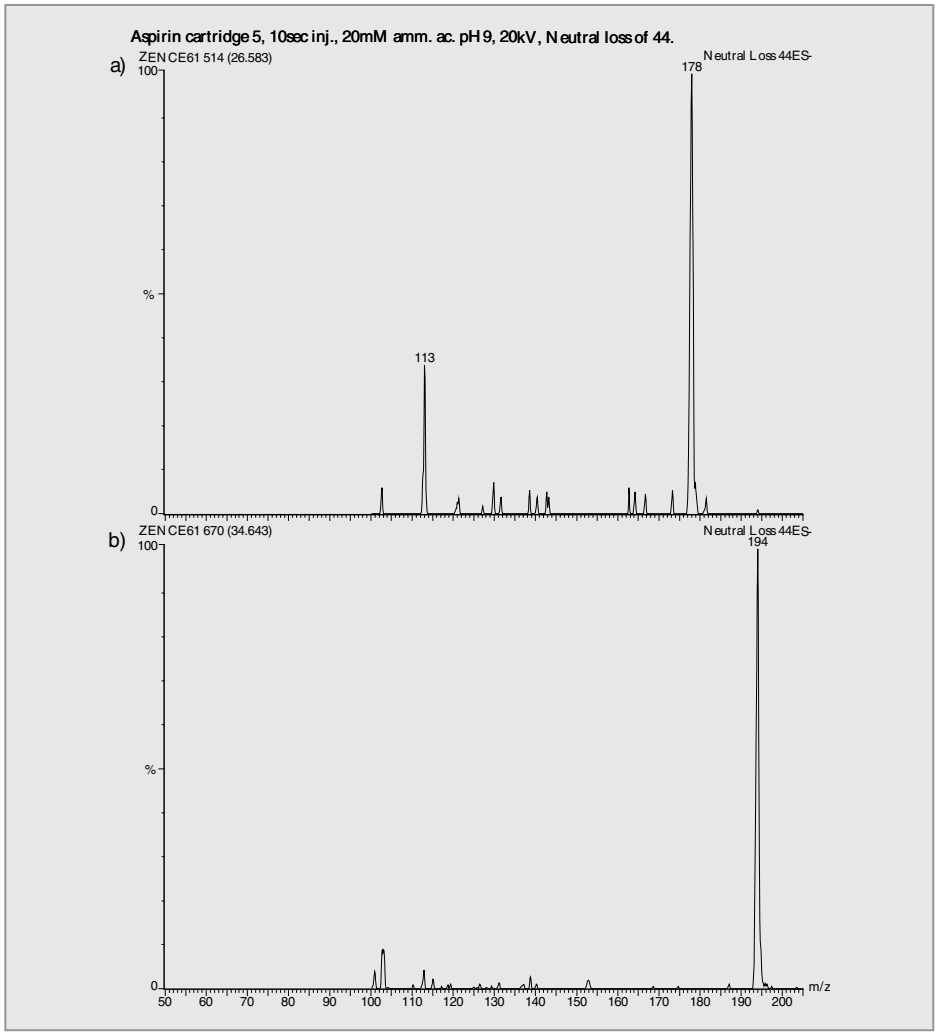

Figure 10. On-line CE-ES-MS-MS Constant Neutral Loss analysis monitoring ions which lose 44 amu:

(a) hippuric acid (MW 179), and

(b) salicylhippuric acid (MW 195).

Summary

These studies show that CE coupled to negative ionization ES-MS can be applied to the detection and identification of a range of non-steroidal anti-inflammatory drugs and their metabolites in urine, with minimal sample clean-up necessary.

Molecular ion species (i.e. $(M-H)^-$) ions were generated to confirm molecular weights, and in addition the use of in-source collision induced dissociation and low energy tandem mass spectrometry facilitated identification of the metabolites produced. Selective screening of the urine extracts for the presence of ibuprofen glucuronide metabolites was achieved by on-line CE-ES-MS-MS monitoring the precursor ions of the characteristic dehydrated glucuronic acid ion.

The system used was operated for several days without any maintenance, due in part to the fact that CE requires only minimum amounts of dirty matrices to be injected. An environmental advantage of this technique over LC-MS methods is that solvent consumption is minimized.

The results for each analysis were generated by injecting only 30 nL of the available, reconstituted urine samples, which were collected following standard therapeutic doses of the drugs. The ability to detect metabolites in this way could have a significant impact in the determination of such substances in biological matrices for both drug metabolism studies and in areas such as forensic toxicology and the detection of drugs of abuse.

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