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Evaluation of liquid chromatography–ion spray mass spectrometry for the determination of substituted benzoic acids and their glycine conjugates☆

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Abstract

Liquid chromatography combined with pneumatically assisted electrospray (ion spray) mass spectrometry was evaluated for the characterization of low-molecular-mass compounds such as a variety of substituted benzoic acids and their glycine conjugates. Each substituted benzoic acid and its glycine conjugate formed with rat liver mitochondria were separated with a conventional C_8 packed column or a semimicro C_8 column using three mobile phases. The positive- and negative-ion mass spectra of the glycine conjugates gave abundant $[M+H]^+$ and $[M-H]^-$ ions, respectively. However, the positive-ion mass spectra of the acids were dominated by $[M+H-H_2O]^+$ ions, with the exception of $[M+H]^+$ ions for acids having an amino, an acetylamino or a dimethylamino group. All of the negative-ion mass spectra of acids gave dominant $[M-H]^-$ ions. The negative-ion mass spectra of acids with halogen, nitro and cyano groups showed the ions equivalent to the mass of each functional group. Further, the alkoxyl group gave a characteristic fragmentation representing loss of the alkyl moiety. The abundances of the fragment ions due to the functional groups gave information on the position of the ring substituents in the negative-ion mass spectra. The positive- and negative-ion mass spectra of the glycine conjugates revealed the presence of glycine.

1. Introduction

Compounds containing the carboxylic acid group are of great significance as drugs, herbicides and insecticides owing to a variety of interesting biological activities. In addition, xenobiotics are readily converted into carboxylic acids by metabolism. A wide range of carboxylic acids can undergo amino acids conjugations, which vary with the animal species and the structure of the acids. Glycine conjugation has been observed to be a feature of a diversity of carboxylic acids in a wide range of species. Correlations between the chemical structure of acids and glycine conjugation are also important in order to understand detoxification mechanisms and have been discussed [1–4]. We have also elucidated the influence of chemical structure on the extent of glycine conjugation [5–7], which required methods for the determination of these compounds. Conventional gas chromatography–mass spectrometry (GC–MS) has been

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☆ This paper is dedicated to Professor Yasumitsu Tamura on the occasion of his 70th birthday.

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widely applied to the determination of the glycine conjugates. However, no information is available about pneumatically assisted liquid chromatography–electrospray mass spectrometry (LC–ion spray MS), which has been used to detect and identify a series of organic acids and their glycine conjugates. In addition, there is no report about their spectral features. It is useful for metabolism studies to elucidate the spectral characteristics of a series of acids and their glycine conjugates using different analytical techniques.

Liquid chromatography–mass spectrometry (LC–MS), which permits the separation and ionization of polar, non-volatile or thermally labile compounds without derivatization, has been widely applied in various analytical biomedical and environmental fields. Recent advances in electrospray ionization (ESI) have expanded the capabilities of LC–MS. Using this ionization technique, high-molecular-mass compounds such as proteins and peptides have been ionized and measured [8,9]. ESI has shown superior performance to other ionization methods in biomolecular analysis. In addition, the technique has allowed detection of very polar and ionic compounds. Another advantage of ESI is the low chemical background. We have previously characterized various substituted benzoic acids and their glycine conjugates by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS) [10]. However, the acids and their glycine conjugates have very low molecular masses. Therefore, it was difficult to measure and identify lower concentrations of acids and their glycine conjugates by LC–APCI-MS owing to the higher chemical background. ESI has an excellent capability for application to very low-molecular-mass compounds because of its intrinsically low background.

Ionization based on the ion evaporation mechanism is known to be very soft and thus minimizes the fragmentation. Therefore, molecular mass information is readily accessible from an ion evaporation mass spectrum [11]. However, the mass spectra reveal no structural information. If extra internal energy is added to the ions, fragmentation can be promoted. Tandem mass spectrometry (MS–MS) is well known for the structural information that it can provide in conjunction with soft ionization techniques [12,13]. On the other hand, it is necessary for structural identification to evaluate the use of collision-induced dissociation (CID) in combination with a single MS technique.

This paper reports the evaluation of LC–ion spray MS for the determination of a series of acids and their glycine conjugates. The extent to which degree structural information is gained by CID is also discussed. In addition, the mass spectrometric fragmentation properties of acids and their glycine conjugates are described.

2. Experimental

2.1. Chemicals

Benzoic acid and 2-methoxy-, 2-nitro-, 3-amino-, 3-chloro-, 3-methoxy-, 3-methyl-, 3-nitro-, 4-amino-, 4-acetylamino-, 4-bromo-, 4-chloro-, 4-cyano-, 4-ethoxy-, 4-methoxy-, 4-methyl-, 4-nitro-, 4-dimethylamino-, 2-chloro-4-nitrobenzoic acid were purchased from Nacalai Tesque (Kyoto, Japan). Hippuric acid and 4-aminohippuric acid were purchased from Wako (Osaka, Japan). All other chemicals were of analytical-reagent grade. N-(4-Acetylaminobenzoyl)glycine was synthesized by the method described previously [5].

2.2. Preparation of mitochondria

The animals used were Wistar-strain male rats weighing about 200–250 g. Mitochondria were prepared by the method described previously [6].

2.3. Formation of glycine conjugates of a series of substituted benzoic acids

The formation of glycine conjugates was carried out by using the technique described previously [6,7].

2.4. Instrumentation

HPLC separation was performed with a Hitachi (Tokyo, Japan) L-6200 instrument having a 5-μm Cosmosil C_8 reversed-phase column

(150 mm × 4.6 mm I.D.) (Nacalai Tesque) or a 5-μm Develosil C_8 reversed-phase semimicro column (150 mm × 1.5 mm I.D.) (Nomura Chemical, Aichi, Japan). The mobile phases consisted of water–methanol–acetic acid systems as follows: (a) 49.8:50:0.2, (b) 61.3:38.5:0.2 and (c) 77.8:22:0.2 (v/v/v). The flow-rate was 1 ml/min or 50 μl/min. Each substituted benzoic acid and its glycine conjugate were separated using the same mobile phases as described previously [10]. A Hitachi M-2000 double-focusing mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source was used. The interface for introducing the HPLC effluent to the APCI-MS system was pneumatically assisted electrospray. Analyte ions formed from the LC–ion spray MS interface were sampled into the mass spectrometer through 0.2- and 0.4-mm orifices. All positive- and negative-ion mass spectral data were obtained by scanning the mass range from *m/z* 1 to 500 in 4 s, with a dwell time of 0.5 s. The drift voltage varied in the range 30–75 V.

3. Results and discussion

In previous work we studied the mass spectral characterization of substituted benzoic acids and their glycine conjugates by LC–APCI-MS [10]. LC–APCI-MS provided some structural and molecular mass information. LC–ion spray MS–MS has recently been applied as an extremely attractive method for the characterization of proteins and peptides. On the other hand, the advantage of LC–ion spray MS is the low chemical background. Therefore, we used LC–ion spray MS for the determination of low-molecular-mass compounds such as substituted benzoic acids and their glycine conjugates. The ion formation process for ion spray is different from that for APCI. The ionization mechanism in APCI is known to be very similar to that of CI, whereas that of ESI is ion evaporation. As ion evaporation is a very soft ionization process, the molecular mass can be easily determined. However, the molecular mass information alone is not sufficient for structural identification of analytes. It is important for the identification of the metabolites to gain some structural information. We investigated the use of CID for LC–ion spray MS analysis and the extent to which structural information was provided from CID mass spectra in a single MS mode.

The positive- and negative-ion mass spectra of benzoic acid derivatives were measured using water–methanol (50:50, v/v) as the eluent at a flow-rate of 50 μl/min in the flow-injection mode (no column).

Table 1 shows the effect of drift voltage on the characteristic ions in the negative-ion mass spectra of 2-chloro-4-nitrobenzoic acid. The drift voltage is necessary in order to dissociate cluster ions into a high-abundance deprotonated molecular ion. When extra energy is added to the deprotonated molecular ion, fragmentation can be promoted. At a drift voltage of 30 V, the negative-ion mass spectrum of 2-chloro-4-nitrobenzoic acid gave a dominant $[M-H]^-$ ion at *m/z* 200 with little fragmentation. However, at a drift voltage of 40 V, increases in the signals at *m/z* 156 ($[M-COOH]^-$) and 35 (Cl^-) occurred owing to CID of the deprotonated molecular ion. At drift voltages more than 45 V, the characteristic ion at *m/z* 46 (NO_2^-) was also observed. The ease of elimination of the substituents depends on the position of the ring substituent. Even if the substituent is present in the same *para* position on the benzene ring, the degree of elimination differs in the type of the substituent. The ions equivalent to the mass of the iodo and bromo groups in the *para* position were easily observed at a lower drift voltage. At a slightly higher drift voltage, the fragment ion

Table 1
Effect of drift voltage on characteristic ions in the negative-ion mass spectra of 2-chloro-4-nitrobenzoic acid

Drift voltage (V)	Relative ion abundance (%)			
	m/z 200	*m/z* 156	*m/z* 46	*m/z* 35
30	100	8	0	0
40	38	100	0	80
45	30	100	22	100

Mobile phase, water–methanol (50:50, v/v); flow-rate, 50 μl/min; amount injected, 300 ng; injection, flow-injection mode.

due to elimination of the cyano group in the *para* position appeared. The order of elimination of the functional group in the *para* position was Br^-, I^-, $NO_2^- > Cl^- > CN^-$. The drift voltage was kept at 50 V to obtain some structural information.

Table 2 shows characteristic ions of the negative-ion mass spectra of the *para*-substituted benzoic acids. The negative-ion mass spectra were relatively simple. $[M-COOH]^-$ ions were generated by the same fragmentations as observed for all benzoic acid derivatives. The same observations on the fragmentation of 2-chloro-4-nitrobenzoic acid were made for the mass spectra of the acids having a cyano or a halo group. The characteristic ions at *m/z* 26, 79 and 127 indicated the possible presence of cyano, bromo and iodo moieties, respectively. In the mass spectra of 4-methoxy- and 4-ethoxybenzoic acid, important diagnostic ions at *m/z* 92 were observed, which correspond to $[M-COOH-CH_3]^-$ and $[M-COOH-C_2H_5]^-$ ions, respectively. The alkoxyl group gave a characteristic fragmentation representing loss of the alkyl moiety. An alkyl ion in the low-mass range did not appear. In contrast, the mass spectra of 4-methyl-, 4-amino- and 4-dimethylaminobenzoic acid were very simple, consisting primarily of $[M-H]^-$ and $[M-COOH]^-$ ions. At a high drift voltage of 75 V, the ion due to the cleavage of the substituent was not observed. In addition, the negative-ion detection for acids having an amino, an acetylamino or a dimethylamino group decreased compared with that for other acids.

Table 3
Characteristic ions of the positive-ion mass spectra of 4-methyl- and 4-aminobenzoic acid

Substituent	Molecular mass	Characteristic ions[a]	
		$[M+H]^+$	$[M+H-H_2O]^+$
4-CH_3	136	–	119 (100)
4-NH_2	137	138 (100)	120 (76)

Mobile phase, water–methanol (50:50, v/v); flow-rate, 50 μl/min; amount injected, 500 ng; injection, flow-injection mode.
[a] Values are *m/z* with relative intensities (%) in parentheses.

Table 3 shows characteristic ions of the positive-ion mass spectra of 4-methyl- and 4-aminobenzoic acid. The positive-ion mass spectra of the substituted benzoic acids were different from the negative-ion mass spectra. The positive-ion mass spectra of benzoic acid derivatives with an amino, an acetylamino or a dimethylamino group gave dominant $[M+H]^+$

Table 2
Characteristic ions of the negative-ion mass spectra of the substituted benzoic acids

Substituent	Molecular mass	Characteristic ions[a]			
		$[M-H]^-$	$[M-COOH]^-$	$[M-COOH-R^b]^-$	$[X^c]^-$
4-CN	147	146 (40)	102 (100)		26 (9)
4-Cl	156	155 (71)	111 (100)		35 (16)
4-Br	200	199 (63)	155 (100)		79 (38)
4-I	248	247 (100)	203 (89)		127 (72)
4-OCH_3	152	151 (51)	107 (100)	92 (12)	
4-OC_2H_5	166	165 (80)	121 (100)	92 (24)	
4-CH_3	136	135 (79)	91 (100)		
4-NH_2	137	136 (83)	92 (100)		
4-$N(CH_3)_2$	165	164 (51)	120 (100)		

Mobile phase, water–methanol (50:50, v/v); flow-rate, 50 μl/min; amount injected, 300 ng; injection, flow-injection mode. The drift voltage was kept at 65 V for 4-cyano-, 4-methoxy and 4-ethoxybenzoic acid.
[a] Values are *m/z* with relative intensities (%) in parentheses.
[b] R = CH_3 and C_2H_5.
[c] X = CN, Cl, Br and I.

ions and $[M+H-H_2O]^+$ ions, whereas the positive-ion mass spectra of all the series except for these acids were dominated by $[M+H-H_2O]^+$ ions.

The influence of the position of the ring substituent on the mass spectrum was also investigated. Table 4 shows the influence of the position of the nitro group on the nitro anion abundance in the negative-ion mass spectra of nitrobenzoic acid. At a drift voltage of 50 V, no significant differences in the ion abundances at *m/z* 46 were observed. However, at a drift voltage of 45 V, the ion abundances at *m/z* 46 increased in the order 4-position < 3-position < 2-position (on the benzene ring). On the other hand, the mass spectra of the acids having a methoxy group offered different fragment ions in correlation with the position of the substituent (Table 4). In the mass spectrum of 4-methoxybenzoic acid taken at a drift voltage of 65 V, a signal at *m/z* 92 corresponding to $[M-COOH-CH_3]^-$ was observed. With the methoxy group in the 2-position, the signal at *m/z* 92 disappeared, whereas that at *m/z* 77 corresponding to $[M-COOH-OCH_3+H]^-$ increased compared with the 3- and 4-positions. The fragment ions due to elimination of the substituent gave information on the position of the substituent.

Glycine conjugation was dependent not only on the lipophilicity of the chemicals but also on the size of both the *para* and *meta* substituents on the benzene ring. The acids having chlorine, methyl, methoxy and ethoxy groups in either the *para* or *meta* position on the benzene ring showed high glycine conjugate formation. The acids bearing cyano, nitro, amino, acetylamino and dimethylamino groups in the *para* or *meta* position resulted in decreases in glycine conjugation. On the other hand, the acids having a substituent in the *ortho* position were conjugated with glycine to only a small extent or did not undergo glycine conjugation.

Three synthetic glycine conjugates available were determined in both the positive- and negative-ion modes. When no synthetic glycine conjugates were available, the glycine conjugates were formed with rat liver mitochondria. For the HPLC separation of each acid and its glycine conjugate, the acetic acid concentration required for the mobile phase was more than 0.2% in water–methanol. Both a conventional packed column (150 mm × 4.6 mm I.D.) and a semimicro packed column (150 mm × 1.5 mm I.D.) were used. On-line LC with pneumatically assisted electrospray was best accomplished with low mobile phase flow-rates. Flow-rates of up to 200 μl/min could be used. However, the optimum flow-rate was ca. 50 μl/min. Therefore, splitting of the LC eluent with the use of the 4.6 mm I.D. column is necessary in order to increase the ionization efficiency and ensure stable liquid drop formation. When HPLC separation was performed with the use of the 4.6 mm I.D.

Table 4
Influence of the position of the functional groups on characteristic ions in the negative-ion mass spectra of methoxy- and nitrobenzoic acid

Substituent	Relative ion abundance (%)						
	m/z 166	*m/z* 122	*m/z* 46	*m/z* 151	*m/z* 107	*m/z* 92	*m/z* 77
2-NO_2	57	100	82				
3-NO_2	65	100	33				
4-NO_2	56	100	12				
2-OCH_3				62	95	0	100
3-OCH_3				45	100	31	3
4-OCH_3				51	100	12	0

Mobile phase, water–methanol (50:50, v/v); flow-rate, 50 μl/min; amount injected, 300 ng; injection, flow-injection mode; drift voltage, 45 V for nitrobenzoic acid and 65 V for methoxybenzoic acid.

column at a flow-rate of 1.0 ml/min, the splitting ratio was 1:20. Most of the samples injected were split to waste. On the other hand, the major advantage of the use of the semimicro column is that it does not require a postcolumn split. The losses of samples by splitting of the LC eluent could be circumvented by the use of the semimicro column. An increase in sensitivity could be achieved by using the semimicro column when the sample amounts injected were limited. However, larger volumes (20 μl) of samples injected into the semimicro packed column resulted in broader peaks. Separation with the use of the semimicro column was performed using the same mobile phases as with the 4.6 mm I.D. column.

Fig. 1 shows full-scan total ion and extracted ion profiles of the supernatant obtained after incubation with (A) 3-methylbenzoic and (B) 3-chlorobenzoic acid in the rat liver mitochondria. The mass spectra were measured using water–methanol–acetic acid (49.8:50:0.2, v/v/v) as the eluent for both (A) and (B) at a flow-rate of 50 μl/min through a semimicro column. A typical mass chromatogram measured in the positive-ion mode is shown in (A) and that in the negative-ion mode in (B). Inspection of the total ion current (TIC) profile for the positive-ion mode revealed a few chromatographic peaks. The extracted ion profiles for the ions at m/z 119 and 194, corresponding to $[M + H - H_2O]^+$ and $[M + H]^+$, respectively, showed 3-methylbenzoic acid and its glycine conjugate, respectively. The TIC profile for the negative-ion mode was noisy. One reason is that stable liquid drop formation in the negative-ion mode is more difficult than that in the positive-ion mode. The negative-ion mode of operation was less sensitive than the positive-ion detection mode. The presence of 3-chlorobenzoic acid and its glycine conjugate in the rat liver mitochondria could be detected via the extracted ion profiles at m/z 155 and 212, respectively. Characteristic ions of the mass spectra of the glycine conjugates of 3-chlorobenzoic and 3-methylbenzoic acid are shown in Tables 5 and 6. In the negative-ion mode, 100-pmol levels of the acids yielded sufficient $[M - H]^-$ and $[M - COOH]^-$ ion intensities to allow interpretation. Low-molecular-mass compounds such as acids and their glycine conjugates were detected less sensitively by using LC–ion spray MS than in the LC–ion spray MS–MS analysis of proteins reported previously [14]. One reason is that scattering losses in the interface are most likely for ions of relatively low mass. Scattering losses are expected to be less important as the ions become heavier [14]. However, LC–ion spray MS was sufficiently sensitive to be applicable to the detection and identification of acids and their glycine conjugates in rat liver mitochondria.

To obtain structural characteristic information on glycine conjugates, the negative-ion mass spectra of hippuric acid were measured at different drift voltages. The negative-ion mass spectrum of hippuric acid taken at a drift voltage of

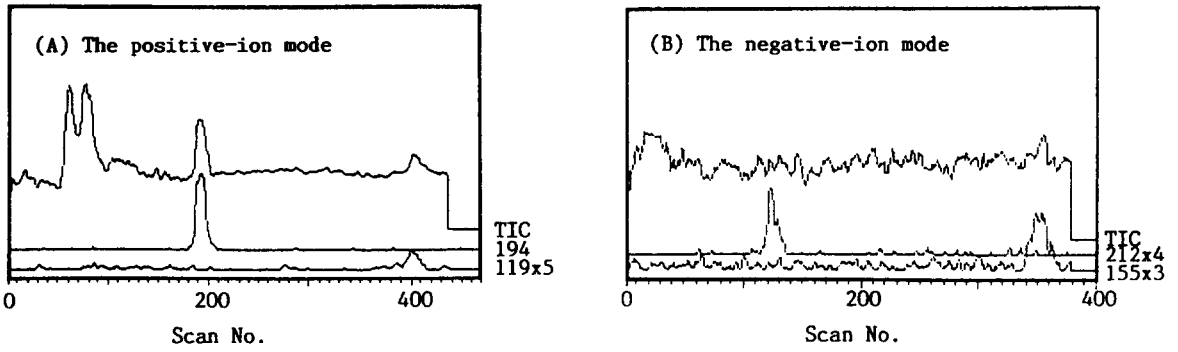

Fig. 1. Typical full-scan total ion and extracted ion profiles of the supernatant obtained after incubation with (A) 3-methylbenzoic acid and (B) 3-chlorobenzoic acid in rat liver mitochondria. Mobile phase, water–methanol–acetic acid (49.8:50:0.2, v/v/v); flow-rate, 50 μl/min.

Table 5
Characteristic ions of the negative-ion mass spectra of glycine conjugates

Substituent	Molecular mass	Characteristic ions[a]			
		$[M-H]^-$	$[M-COOH]^-$	$[M-CONHCH_2COOH]^-$	Others
H	179	178 (100)	134 (46)	77 (66)	
3-Cl	213	212 (80)	168 (55)	111 (60)	35 (100)
4-Cl	213	212 (100)	168 (51)	111 (61)	35 (88)
3-CH_3	193	192 (100)	148 (29)	91 (82)	
4-CH_3	193	192 (100)	148 (41)	91 (53)	
4-CH_3O	209	208 (100)	164 (39)	107 (71)	
4-NH_2	194	193 (100)	149 (43)	92 (71)	
4-CH_3CONH	236	235 (100)	191 (41)	134 (80)	92 (18)

Column, Develosil $5C_8$; drift voltage, 65 V; flow-rate, 50 μl/min; mobile phase, water–methanol–acetic acid (49.8:50:0.2, v/v/v) for the glycine conjugates of 3-chloro-, 4-chloro-, 3-methyl- and 4-methylbenzoic acid, (61.3:38.5:0.2, v/v/v) for the glycine conjugates of benzoic acid and 4-methoxybenzoic acid and (77.8:22:0.2, v/v/v) for the glycine conjugates of 4-amino- and 4-acetylaminobenzoic acid.

[a] Values are m/z with relative intensities (%) in parentheses.

30 V showed a dominant $[M-H]^-$ ion at m/z 178 with very low-abundant fragment ions. The mass spectrum at a drift voltage of 50 V gave only abundant $[M-H]^-$ and $[M-COOH]^-$ ions, which were observed in the negative-ion mass spectra of the acids. At a drift voltage of 75 V, an additional signal at m/z 77 ($[M-CONHCH_2COOH]^-$) increased (Table 5). The drift voltage was set to 65 V in order to promote specific fragmentations of glycine conjugate. Table 5 shows characteristic ions in the negative-ion mass spectra obtained from tracing the deprotonated molecular ions of glycine conjugates. The mass spectra were measured using three mobile phases at a flow-rate of 50 μl/min through a reversed-phase semimicro column. All negative-ion mass spectra gave characteristic ions of the glycine conjugates, consisting of $[M-H]^-$, $[M-COOH]^-$ and $[M-CONHCH_2COOH]^-$ ions. The mass spectrum of

Table 6
Characteristic ions of the positive-ion mass spectra of glycine conjugates

Substituent	Molecular mass	Characteristic ions[a]			
		$[M+H]^+$	$[M-NHCH_2COOH]^+$	$[M-CONHCH_2COOH]^+$	Others ($[M+Na]^+$ etc.)
H	179	180 (48)	105 (100)	77 (16)	202 (10)
3-Cl	213	214 (62)	139 (100)	111 (14)	236 (8)
4-Cl	213	214 (55)	139 (100)	111 (10)	236 (12)
3-CH_3	193	194 (21)	119 (100)	91 (17)	216 (13)
4-CH_3	193	194 (18)	119 (100)	91 (11)	216 (18)
4-CH_3O	209	210 (20)	135 (100)		232 (9), 121 (10)
4-NH_2	194	195 (26)	120 (100)	92 (26)	217 (10)
4-CH_3CONH	236	237 (35)	162 (100)		259 (16), 120 (63)

Column, Develosil $5C_8$; drift voltage, 65 V; flow-rate, 50 μl/min; mobile phase, water–methanol–acetic acid (49.5:50:0.2, v/v/v) for the glycine conjugates of 3-chloro-, 4-chloro-, 3-methyl- and 4-methylbenzoic acid, (61.3:38.5:0.2, v/v/v) for the glycine conjugates of benzoic acid and 4-methoxybenzoic acid and (77.8:22:0.2, v/v/v) for the glycine conjugates of 4-amino- and 4-acetylaminobenzoic acid.

[a] Values are m/z with relative intensities (%) in parentheses.

4-acetylaminohippuric acids showed an additional signal at m/z 92, corresponding to a characteristic loss of CH_2CO from the $[M - CONHCH_2COOH]^-$ ion. In the presence of a chloro group, the chloride anion was observed in the low-mass region. The ions due to the cleavage of the substituent were also observed in the same fragmentation as with the acids.

The positive-ion mass spectra were determined by the same method as for the negative-ion mode. In the mass spectrum of the glycine conjugate of 4-aminobenzoic acid, a drift voltage of 30 V led to a dominant $[M + H]^+$ ion, but a change from 40 to 75 V led to higher fragmentation levels, resulting in increases in signals at m/z 120 ($[M - NHCH_2COOH]^+$) and 92 ($[M - CONHCH_2COOH]^+$). These fragmentations in the positive-ion mass spectra were characteristic for all glycine conjugates. However, a signal corresponding to loss of CH_2COOH from the protonated molecular ion, which is one of characteristic ions for the glycine conjugates obtained by LC–APCI-MS, could not be observed in the ion spray mass spectrum. The positive-ion mass spectra of glycine conjugates provided more useful structural information for the presence of glycine than the negative-ion mass spectra. Apart from these fragment ions, the mass spectrum of 4-acetylaminohippuric acid taken at a drift voltage of 75 V showed $[M - NHCH_2COOH - CH_3CO + H]^+$ ion at m/z 120. In the presence of an alkyl or an amino group, no information on the substituents was obtained in either the positive- or the negative-ion mode. The chloride anion also was not observed in the low-mass region.

4. Conclusions

LC–ion spray MS was applied to the determination of low-molecular-mass compounds such as benzoic acid derivatives and their glycine conjugates. LC–ion spray MS could make interpretation of the spectrum obtained from low levels of the analyte easier than with LC–APCI-MS. LC–ion spray MS did not always provide molecular information. All of the negative-ion mass spectra of the substituted benzoic acids and their glycine conjugates gave dominant $[M - H]^-$ ions with little fragmentation. However, all of the positive-ion mass spectra of glycine conjugates were dominated by $[M + H]^+$ ions, whereas those of acids gave dominant $[M + H - H_2O]^+$ ions, except for the $[M + H]^+$ ions for the acids having an amino, an acetylamino or a dimethylamino group. LC–ion spray MS provided less structural information than LC–APCI-MS. However, the CID technique was beneficial for obtaining some structural information on the substituted benzoic acids and their glycine conjugates even using the single MS mode. The negative-ion mass spectra of the substituted benzoic acids permitted identification of the position and kind of substituent. The negative- and positive-ion mass spectra of glycine conjugates revealed the presence of glycine and the kind of substituent from the characteristic fragmentation patterns. The mass spectrometric fragmentation properties obtained from LC–ion spray MS allow the detection and identification of metabolites in biological samples.

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