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Liquid chromatographic–atmospheric pressure chemical ionization mass spectral characterization of carboxylic acids and their glycine conjugates

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ABSTRACT

A series of substituted benzoic acids and their glycine conjugates were characterized by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. Each substituted benzoic acid and its glycine conjugate were separated with a Cosmosil 5 C_8 column using three mobile phases. All negative-ion mass spectra of the substituted benzoic acids gave abundant $[M-H]^-$ and $[M-COOH]^-$ ions. For acids with halogen, nitro, cyano, and acetylamino groups, fragmentations corresponding to losses equivalent to the mass of each functional group were also observed. In addition, 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. All of the positive- and negative-ion mass spectra of the glycine conjugates gave abundant $[M+H]^+$ and $[M-H]^-$ ions with characteristic fragment ions, respectively. Fragmentations of the glycine conjugates obtained in the positive-ion mode were different from those in the negative-ion mode. Similar information on the substituents was obtained for the glycine conjugates.

INTRODUCTION

Xenobiotics containing carboxylic acid groups are very commonly encountered and are of great significance as drugs, herbicides, plant hormones and insecticides. The carboxylic acid group may also result from metabolism of a xenobiotic. Glycine conjugation is the most important route in the metabolism of carboxylic acids. Correlations between molecular structure and enzymatic activities are extremely important in order to understand detoxification mechanisms. Therefore, relationships between the chemical structure of acids and glycine conjugation have been investigated [1–4]. We have also studied a series of substituted benzoic acids as simple model compounds of carboxylic acids and have elucidated the influence of chemical structure on the extent of glycine conjugation [5–7].

For the determination of glycine conjugation activities, we developed a specific and simple high-performance liquid chromatographic (HPLC) method. Because only a few synthetic glycine conjugates are available, enzymatic activities were calculated from the decreasing rates of the substrates. However, it was confirmed that a decrease in the size of the peak of the substrate resulted in a concomitant increase in that of the glycine conjugate. When synthetic glycine conjugates were available, the glycine conjugates were tentatively identified by comparison with the chromatographic properties of the synthetic glycine conjugates. When no synthetic glycine conjugates are available, it is difficult to identify the glycine conjugates formed in mitochondria.

There have been no reports of investigations in which liquid chromatography–mass spec-

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trometry (LC–MS) has been used to identify various glycine conjugates produced from substituted benzoic acids in the liver mitochondria and to define their spectral characteristics. Further, it is useful for metabolism studies to elucidate the spectrometric features of a wide variety of acids. The conventional gas chromatographic–mass spectrometric (GC–MS) analysis for the identification of these compounds requires chemical derivatization such as methylation or silylation. Recently, LC–MS has played an increasingly important role in a variety of biochemical fields. One of the main advantages of LC–MS is that polar, non-volatile or thermally stable compounds do not require a derivatization step. This system seems to be very attractive for structural identification in metabolism studies.

This paper reports the determination of a series of benzoic acid derivatives and their glycine conjugates using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS). The mass spectrometric fragmentation properties of the acids and their glycine conjugates are also described.

EXPERIMENTAL

Chemicals

Benzoic acid and 2-chloro-, 2-methoxy-, 2-nitro-, 3-amino-, 3-acetylamino-, 3-chloro-, 3-methoxy-, 3-methyl-, 3-nitro-, 4-amino-, 4-acetylamino-, 4-chloro-, 4-cyano-, 4-ethoxy-, 4-methoxy-, 4-methyl-, 4-nitro-, 4-dimethylamino- and 3-nitro-4-chlorobenzoic acid were purchased from Nacalai Tesque (Kyoto, Japan), 3-cyanobenzoic acid from Aldrich (Milwaukee, WI, USA) and hippuric acid and 4-aminohippuric acid from Wako (Osaka, Japan). All other chemicals were of analytical-reagent grade.

3-Butoxy-4-aminobenzoic acid, N-(3-butoxy-4-aminobenzoyl)glycine and N-(4-acetylaminobenzoyl)glycine were synthesized as described previously [5,6].

Preparation of mitochondria

The animals used were ddY strain male mice weighing about 25–30 g. Mitochondria were prepared by the method described previously [6]. Protein concentration was determined by the method of Lowry *et al.* [8].

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].

Instrumentation

HPLC separation was performed with a Hitachi (Tokyo, Japan) L-6200 instrument equipped with a 5-μm Cosmosil C_8 reversed-phase column (150 mm × 4.5 mm I.D.) (Nacalai Tesque). The mobile phases were 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. The mobile phases used for the separation of substituted benzoic acids and their glycine conjugates are given in Table I. The column effluent of each acid and its glycine conjugate was monitored at the wavelength described previously.

Positive- and negative-ion mass spectra were measured with a Hitachi M-2000 double-focusing mass spectrometer equipped with an atmospheric pressure ionization source. All mass spectral data were obtained by scanning the mass range from m/z 1 to 600 in 4 s, with a dwell time of 0.5 s. Sensitivity measurements were carried out with selected ion monitoring (SIM) by flow-injection analysis. The nebulizer temperature

TABLE I

MOBILE PHASES FOR SEPARATION OF SUBSTITUTED BENZOIC ACIDS AND THEIR GLYCINE CONJUGATES

Mobile phase (Water–methanol–acetic acid)	Substituted benzoic acids
(a) 49.8:50:0.2 (v/v/v)	4-Cl-, 4-CH_3-, 4-C_2H_5O-, 4-$(CH_3)_2N$-, 3-Cl-, 3-CH_3-, 3-C_4H_9O-4-NH_2-, 3-NO_2-4-Cl-
(b) 61.3:38.5:0.2 (v/v/v)	H-, 4-CN-, 4-NO_2-, 4-CH_3O-, 3-CN-, 3-NO_2-, 3-CH_3O-
(c) 77.8:22:0.2 (v/v/v)	4-NH_2-, 4-CH_3CONH-, 3-NH_2-, 3-CH_3CONH-

was set at 300 or 340°C. The drift voltage varied in the range 120–170 V.

RESULTS AND DISCUSSION

In a previous study, HPLC separation of a series of acids and their glycine conjugates, tentatively identified, was achieved by using water–methanol–acetic acid–0.5 *M* tetrabutylammonium hydroxide as the mobile phase [6]. However, the mobile phase is restricted in LC–MS methods. As the ion-pair reagent, tetrabutylammonium hydroxide, was not appropriate in the the mobile phase, it was omitted. Although a solvent system of water–methanol–acetic acid was used, an acid and its glycine conjugate, tentatively identified, could be well separated. Retention times (t_R) of the glycine conjugates were shorter than those obtained by using the previous mobile phase, whereas those of the substrates were longer. A decrease in the size of the peak of a substrate and a corresponding increase in that of the glycine conjugate, tentatively identified, were observed on the chromatogram. When authentic glycine conjugates were available, each glycine conjugate could be detected by comparing the retention time with that of the authentic glycine conjugate. However, there were only a few synthetic glycine conjugates available. Therefore, LC–APCI-MS was applied to identify each increasing peak as the glycine conjugate of each acid.

Fig. 1 shows UV detection and extracted ion profiles (full-scan mode) of the supernatant obtained after incubation with (A) 4-methoxybenzoic and (B) 3-chlorobenzoic acid in the mouse liver mitochondria. The UV traces each showed two peaks, respectively. Individual peaks were monitored by the $[M-H]^-$ ions of the acids or their glycine conjugates. Each increasing peak was identified as the glycine conjugate from the mass spectral fragmentation patterns. In addition, the chromatographic properties for a series of acids and their glycine conjugates were expressed as the relative t_R values (t_R of conjugate/t_R of parent acid). When various acids and their glycine conjugates could be separated using mobile phase (a) or (b), the relative t_R values were 0.36 ± 0.02 and were also constant. With mobile phase (c), the relative t_R values were 0.49 ± 0.03. These findings indicate that calculation of the relative t_R value will permit the provisional identification of an unknown conjugate.

The ionization mechanism in APCI is known to be very similar to that of CI, and therefore the molecular mass can be easily determined. However, it is necessary for identification of the metabolites to obtain structural information. The optimum analytical conditions were investigated to obtain some structural information.

The positive- and negative-ion mass spectra of benzoic acid derivatives were measured using water–methanol (50:50, v/v) as the mobile phase at a flow-rate of 1.0 ml/min in the flow-injection mode (no column). Benzoic acid reacts as a strong acid in the gas phase, resulting in the generation of negative ions in the APCI source.

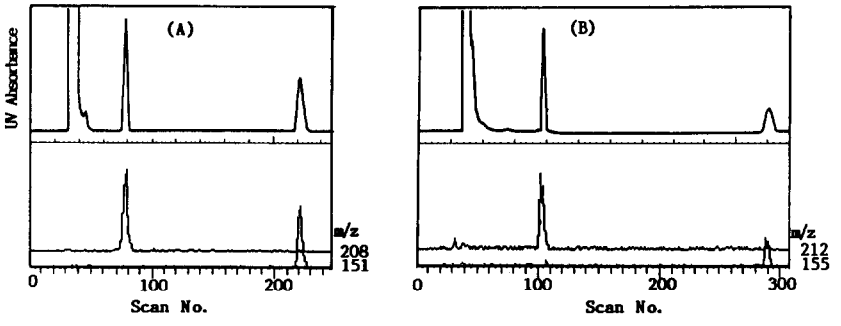

Fig. 1. Typical UV detection and extracted ion profiles of the supernatant obtained after incubation with (A) 4-methoxybenzoic acid and (B) 3-chlorobenzoic acid in mouse liver mitochondria. Detector wavelength, (A) 270 and (B) 235 nm; mobile phase, water–methanol–acetic acid, (A) 61.3:38.5:0.2 and (B) 49.8:50:0.2 (v/v/v).

Therefore, the mass spectra of the substituted benzoic acids could be achieved not in the positive- but in the negative-ion mode. Because of the background due to the mobile phase, it is difficult to obtain good mass spectra at low nebulizer temperatures, so the nebulizer temperature was set at >300°C. As acetic acid was required in the mobile phase for the HPLC separation of an acid and its glycine conjugate, the $[M-H]^-$ responses of the acids having an amino group decreased with 0.2% of acetic acid in water–methanol as the mobile phase. The negative-ion mass spectra obtained were relatively simple.

Fig. 2 shows effect of drift voltage on the negative-ion mass spectra of 4-chlorobenzoic acid. A drift voltage is necessary in order to increase the ionization efficiency and to dissociate cluster ions into a high-abundance deprotonated molecular ion. At a drift voltage of 120 V, the negative-ion mass spectrum of 4-chlorobenzoic acid gave a dominant $[M-H]^-$ ion with low-abundance cluster ions. At drift voltages >130 V, increases in the signals at m/z 111 ($[M-COOH]^-$) and 35 ($[Cl]^-$) occurred owing to collision-induced dissociation of the deprotonated molecular ion. Therefore, the drift voltage was kept at 150 V to obtain some structural information.

Figs. 3 and 4 show the negative-ion mass spectra of 4-cyano-, 3-nitro-4-chloro-, 4-methoxy-, 4-ethoxy-, 3-butoxy-4-amino-, 4-acetylamino-, 4-methyl-, 4-amino- and 4-dimethylaminobenzoic acid. The same fragmentation tendency as for 4-chlorobenzoic acid was observed in the mass spectra of the acids with a cyano or a nitro group. The ions at m/z 46 and 26 indicated the possible presence of the nitro and cyano moieties, respectively. In the mass spectrum of 3-nitro-4-chlorobenzoic acid, two characteristic ions at m/z 35 and 46 were also observed. The mass spectrum of 4-methoxybenzoic acid gave $[M-H]^-$ at m/z 151, $[M-COOH]^-$ at m/z 107 and $[M-COOH-CH_3]^-$ at m/z 92. The mass spectra of 4-ethoxy- and 3-butoxy-4-aminobenzoic acid had important diagnostic ions, which were assigned to $[M-COOH-C_2H_5]^-$ at m/z 93 and $[M-COOH-C_4H_9]^-$ at m/z 107, respectively. The alkoxyl

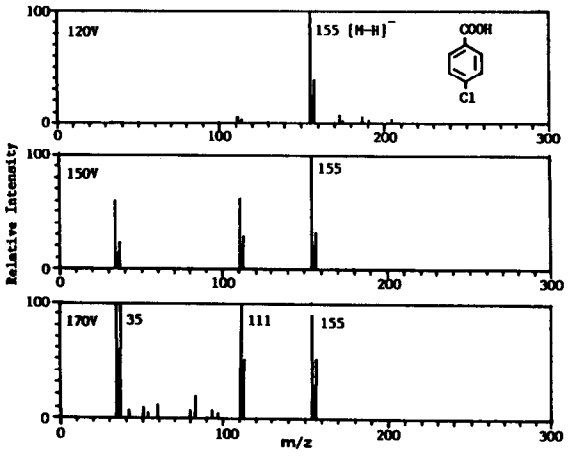

Fig. 2. Effect of drift voltage on the negative-ion mass spectra of 4-chlorobenzoic acid. Mobile phase, water–methanol (50:50, v/v); nebulizer temperature, 300°C; flow-rate, 1 ml/min; injection, flow-injection mode.

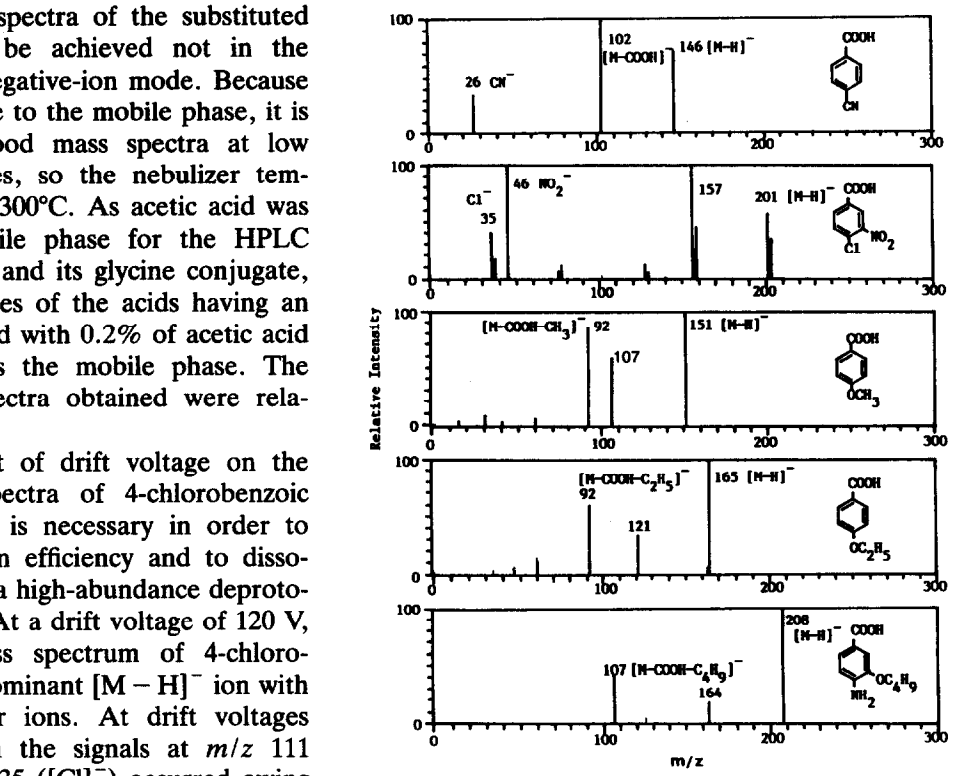

Fig. 3. Negative-ion mass spectra of 4-cyano-, 3-nitro-4-chloro-, 4-methoxy-, 4-ethoxy- and 3-butoxy-4-aminobenzoic acid. Mobile phase, water–methanol (50:50, v/v); nebulizer temperature, 300°C; flow-rate, 1 ml/min; injection, flow-injection mode.

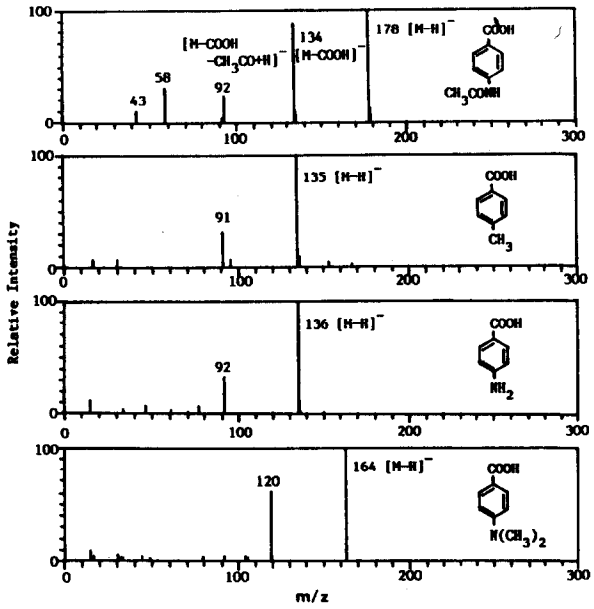

Fig. 4. Negative-ion mass spectra of 4-acetylamino-, 4-methyl-, 4-amino- and 4-dimethylaminobenzoic acid. Mobile phase, water–methanol (50:50, v/v); nebulizer temperature, 300°C; flow-rate, 1 ml/min; injection, flow-injection mode.

group gave a characteristic fragmentation representing loss of the alkyl moiety. Alkyl ions in the low-mass range did not appear. The mass spectrum of 4-acetylaminobenzoic acid gave an $[M-H]^-$ ion at m/z 178 and characteristic ions at m/z 134 ($[M-COOH]^-$), m/z 92 ($[M-COOH-CH_3CO+H]^-$), 58 ($[CH_3CONH]^-$) and 43 ($[CH_3CO]^-$). In contrast, the mass spectrum of 4-methylbenzoic acid was very simple, consisting primarily of $[M-H]^-$ and $[M-COOH]^-$ ions. At a high drift voltage of 170 V, an ion generated by cleavage of the bond between the methyl group and the benzene ring was not observed. In addition, the same observations were made for the mass spectra of the acids having an amino or a dimethylamino group.

The influence of the position of the ring substituent on the mass spectrum was also investigated. Fig. 5 shows the mass spectra of 2-, 3- and 4-nitrobenzoic acid. At a drift voltage of 150 V, no significant differences in the ion abundances at m/z 46 were observed. However, at a drift voltage of 140 V, the ion abundances at m/z 46 were increased in the order 4-position < 3-position < 2-position (on the benzene ring). On

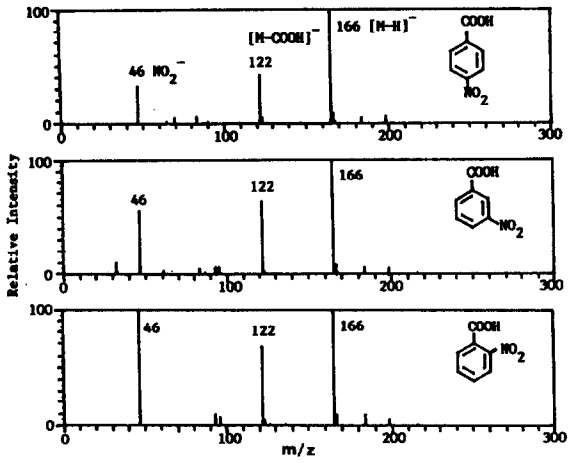

Fig. 5. Influence of the position of the nitro group on the negative-ion mass spectra of nitrobenzoic acid. Mobile phase, water–methanol (50:50, v/v); nebulizer temperature, 300°C; drift voltage, 140 V; flow-rate, 1 ml/min; injection, flow-injection mode.

the other hand, the mass spectra of methoxy-substituted benzoic acids showed different fragment ions depending on the position of the substituent. The negative-ion mass spectra of 2-, 3- and 4-methoxybenzoic acid taken at a drift voltage of 150 V are shown in Fig. 6. In the mass spectrum of 4-methoxybenzoic acid, a signal at m/z 92 corresponding to $[M-COOH-CH_3]^-$

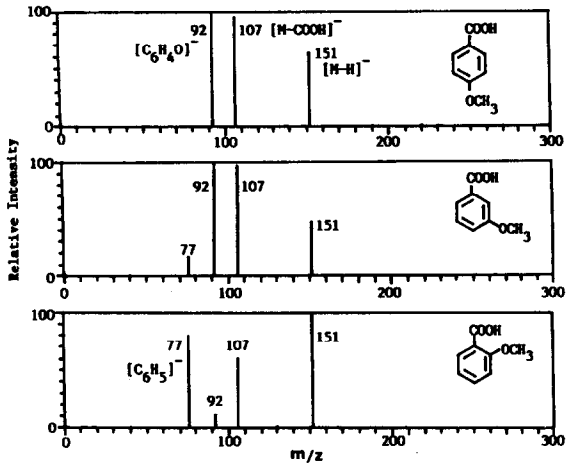

Fig. 6. Influence of the position of the methoxy group on the negative-ion mass spectra of methoxybenzoic acid. Mobile phase, water–methanol (50:50, v/v); nebulizer temperature, 300°C; drift voltage, 150 V; flow-rate, 1 ml/min; injection, flow-injection mode.

was observed. With the methoxy group in the 2-position, the signal at m/z 92 decreased, 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 help to identify the position of the substituent.

The mass spectra of eight glycine conjugates formed in the mouse liver mitochondria and three authentic glycine conjugates could be determined in both the positive- and negative-ion modes. HPLC separation was performed using three mobile phases at a flow-rate of 1.0 ml/min. The acids having the chloro, methyl, methoxy and ethoxy groups in either the *para-* or *meta*-position on the benzene ring showed substantial glycine conjugation. On the other hand, the acids with the cyano, nitro, amino, acetylamino and dimethylamino groups in the *para-* or *meta*-position resulted in decreases in glycine conjugation. However, the acids with the substituent at the *ortho*-position were conjugated with glycine to only a small extent or did not undergo glycine conjugation.

To obtain characteristic fragmentations of glycine conjugates, the mass spectra of 4-aminohippuric acid were taken at different drift voltages with a nebulizer temperature of 340°C. At a drift voltage of 120 V, the mass spectrum gave a dominant $[M - H]^-$ ion at m/z 193 with very low-abundant fragment ions. The mass spectrum at a drift voltage of 150 V gave an abundant $[M - H]^-$ ion with characteristic fragment ions at m/z 149 ($[M - COOH]^-$) and 92 ($[M - CONHCH_2COOH]^-$) (Table II). The drift voltage was kept at 150 V in order to promote specific fragmentations of glycine conjugates. The optimum drift voltage was constant for the nebulizer temperatures in the range 300–340°C.

The negative-ion mass spectra of glycine conjugates of 4-acetylamino-, 4-chloro-, 4-methyl- and 3-butoxy-4-aminobenzoic acid are shown in Fig. 7. All the negative-ion mass spectra gave characteristic ions of the glycine conjugates, consisting of $[M - H]^-$, $[M - COOH]^-$ and $[M - CONHCH_2COOH]^-$ ions. The mass spectra of 4-acetylamino- and 3-butoxy-4-aminohippuric acid showed more abundant additional signals at m/z 92 and 107, corresponding to characteristic losses of CH_2CO and C_4H_9 from $[M - CONHCH_2COOH]^-$ ions, respectively. 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 substituents were also observed in the same fragmentation as with the acids.

Fig. 8 shows the positive-ion mass spectra of the glycine conjugates of 4-methyl-, 4-chloro-, 4-acetylamino- and 3-butoxy-4-aminobenzoic acid. In addition, characteristic ions of the positive-ion mass spectra of glycine conjugates are given in Table III. The positive-ion mass spectra of the glycine conjugates were different from their negative-ion mass spectra, the former

TABLE II

CHARACTERISTIC IONS IN THE NEGATIVE-ION MASS SPECTRA OF GLYCINE CONJUGATES

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

Substituent	Molecular mass	Characteristic ion			
		$[M - H]^-$	$[M - COOH]^-$	$[M - CONHCH_2COOH]^-$	Others
H	179	178 (100)	134 (9)	77 (35)	
3-Cl	213	212 (100)	168 (20)	111 (78)	35 (73)
3-CH_3	193	192 (100)	148 (10)	91 (33)	
3-CH_3O	209	208 (100)	164 (19)	107 (60)	92 (41)
4-CH_3O	209	208 (100)	164 (10)	107 (51)	92 (52)
4-NH_2	194	193 (100)	149 (4)	92 (21)	
4-C_2H_5O	223	222 (100)	178 (12)	121 (48)	92 (59)

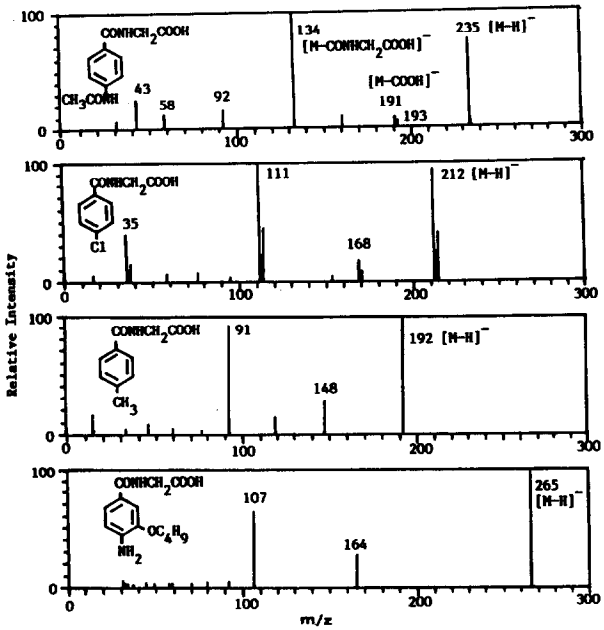

Fig. 7. Negative-ion mass spectra of the glycine conjugates of 4-acetylamino-, 4-chloro-, 4-methyl- and 3-butoxy-4-aminobenzoic acid. Column, Cosmosil $5C_8$; nebulizer temperature, 300–340°C; drift voltage, 150 V; flow-rate, 1 ml/min; mobile phase, see Table I.

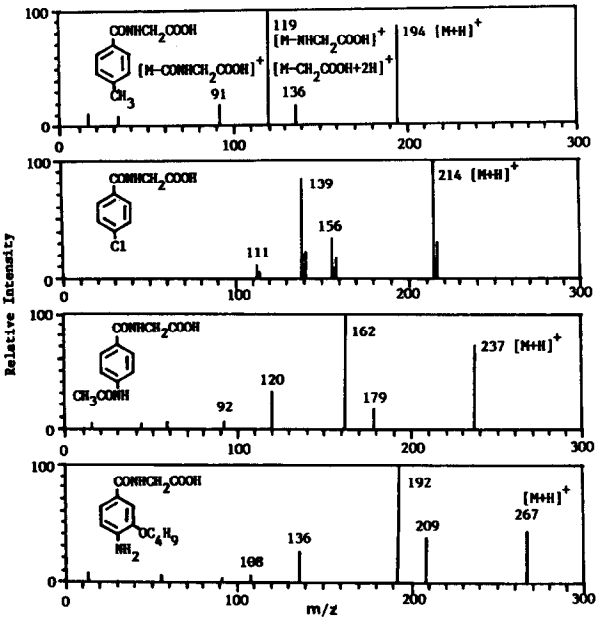

Fig. 8. Positive-ion mass spectra of the glycine conjugates of 4-methyl-, 4-chloro-, 4-acetylamino- and 3-butoxy-4-aminobenzoic acid. Column, Cosmosil $5C_8$; nebulizer temperature, 300–340°C; drift voltage, 150 V; flow-rate, 1 ml/min; mobile phase, see Table I.

providing more useful structural information for the presence of glycine. The mass spectrum of the glycine conjugate of 4-methylbenzoic acid taken at a drift voltage of 150 V gave a dominant $[M+H]^+$ ion at m/z 194 with fragment ions, which were assigned to $[M-CH_2COOH+2H]^+$ at m/z 136, $[M-NHCH_2COOH]^+$ at m/z 119 and $[M-CONHCH_2COOH]^+$ at m/z 91. This fragmentations in the positive-ion mass spectra were characteristic for all glycine conjugates. Apart from these fragment ions, the mass spectrum of 4-acetylaminohippuric acid showed $[M-NHCH_2COOH-CH_3CO+H]^+$ at m/z 120 and $[M-CONHCH_2COOH-CH_3CO+H]^+$ at m/z 92. In the mass spectrum of 3-butoxy-4-aminohippuric acid, more abundant ions at m/z 136 and 108 were also seen, which correspond to loss of a C_4H_8 moiety from $[M-NHCH_2COOH]^+$ and $[M-CONHCH_2COOH]^+$ ions, respectively. The mass spectra of glycine conjugates having an alkoxyl group gave additional fragment ions generated by loss of an alkyl moiety from $[M-NHCH_2COOH]^+$ and $[M-CONHCH_2COOH]^+$ ions, respectively. In the presence of an alkyl or an amino group, no information on the substituents was obtained in either the positive- or negative-ion mode. The chloride anion was not observed in the low-mass region of the positive-ion mass spectra.

The detection limits for a series of substituted benzoic acids and three authentic glycine conjugates were obtained by measuring each deprotonated molecular ion in the SIM mode. The detection limits based on a signal-to-noise ratio of 3:1 were 3.2–36 pmol.

CONCLUSION

All negative-ion mass spectra of the substituted benzoic acids and their glycine conjugates gave dominant $[M-H]^-$ ions with little fragmentation. In addition, similar observations were made for the positive-ion mass spectra of glycine conjugates (the abundant $[M+H]^+$ ions in these cases). However, collision-induced dissociation caused structurally characteristic frag-

TABLE III

CHARACTERISTIC IONS IN THE POSITIVE-ION MASS SPECTRA OF GLYCINE CONJUGATES

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

Substituent	Molecular mass	Characteristic ion				
		$[M+H]^+$	$[M-CH_2COOH+2H]^+$	$[M-NHCH_2COOH]^+$	$[M-CONHCH_2COOH]^+$	Others
H	179	180 (100)	122 (15)	105 (90)	77 (16)	
3-Cl	213	214 (100)	156 (31)	139 (85)	111 (12)	
3-CH_3	193	194 (90)	136 (18)	119 (100)	91 (22)	
3-CH_3O	209	210 (89)	152 (19)	135 (100)		121 (15), 93 (2)
4-CH_3O	209	210 (91)	152 (10)	135 (100)		121 (21), 93 (3)
4-NH_2	194	195 (100)	137 (8)	120 (96)	92 (23)	
4-C_2H_5O	223	224 (100)	166 (12)	149 (100)		121 (19), 93 (2)

mentations. The negative-ion mass spectra of the substituted benzoic acids permitted provisional identification of the position and kind of the 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. Mass spectral features of a variety of acids and their glycine conjugates are suitable for the detection and identification of metabolites in biological samples.

REFERENCES

1 J. Caldwell, in A. Aitio (Editor), *Conjugation Reactions in Drug Biotransformation*, Elsevier/North-Holland, Amsterdam, 1978, p. 111.
2 J. Caldwell, J.R. Idle and R.L. Smith, in T.E. Gram (Editor), *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*, SP Medical and Scientific Books, New York, 1980, p. 453.
3 J. Caldwell, in W.B. Jakoby (Editor), *Metabolic Basis of Detoxication*, Academic Press, New York, 1982, p. 271.
4 H.V. de Waterbeemd, B. Testa and J. Caldwell, *J. Pharm. Pharmacol.*, 38 (1986) 14.
5 F. Kasuya, K. Igarashi and M. Fukui, *J. Pharm. Sci.*, 76 (1987) 303.
6 F. Kasuya, K. Igarashi and M. Fukui, *J. Pharmacobio-Dyn.*, 13 (1990) 432.
7 F. Kasuya, K. Igarashi and M. Fukui, *J. Pharmacobio-Dyn.*, 14 (1991) 671.
8 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265.