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Analysis of β-lactam antibiotics by high performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry using bromoform

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Abstract

The novel identification method for a heat-unstable antibiotic, FC/TA-891 and its active metabolite (FCE22101) by high-performance liquid chromatography (HPLC)–atmospheric pressure chemical ionization mass spectrometry (APCI-MS) employing bromoform as an ionization acceleration solvent, was applied to eight penicillins and 13 cephalosporins which are groups of β-lactam antibiotics. The conditions of HPLC–APCI-MS were examined with ampicillin. Bromoform or chloroform was added to the mobile phase in HPLC to compare the difference between bromine and chlorine adducted ions. For all penicillins except sulbenicillin, both chlorine adducted and bromine adducted ions were observed with a flow injection method. The results indicated that the relative sensitivity ratios of bromine adducted ions to $[M-H]^-$ were higher than those of chlorine adducted ions. These bromine adduct ions could be clearly distinguished from other ions due to its isotopical ratio (1:1), leading to an easy identification of the compounds. For 13 cephalosporins, bromine adducted ions were detected in nine compounds, and chlorine adducted ions were detected in four compounds. The separation of four antibiotics was investigated with an HPLC column to apply this technique to the actual analysis. The capability was equal as in the flow injection method and it found that this technique, i.e. APCI-MS with bromoform could be applicable in the separation analysis.

Keywords: Antibiotics; HPLC–APCI-MS; Chloroform; Bromoform

1. Introduction

High-performance liquid chromatography-mass spectrometry (HPLC-MS) plays an important role in characterizing nonvolatile compounds. There is a strong emphasis for obtaining structural information from HPLC-MS techniques to aid in structural elucidation. HPLC-MS techniques provide ample information for the thermally labile and nonvolatile β-lactam antibiotics.

β-lactam antibiotics, such as deacetoxycephalosporin G, cefradine, produced both informative

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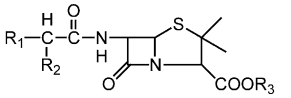
positive and negative mass spectra using HPLC-thermospray-MS [1]. Penicillins and cephalosporins which are groups of β-lactam antibiotics, also produced informative mass spectra and could be determined in bovine milk by using HPLC-electrospray (ESI)-MS [2–4]. However, in the HPLC–APCI-MS, β-lactam antibiotic was rarely investigated to produce informative mass spectra because β-lactam antibiotics were often decomposed in the high source temperature of the APCI interface. As antibiotics, only the results for tetracyclines [5,6] and macrocyclic antibiotic [7] have been reported. For measuring β-lactam antibiotics with HPLC–APCI-MS, new soft ionization methods were required.

Polyols in uremic serum obtained from haemodialysis patients were successfully analyzed with HPLC–APCI-MS using 1% chloroform–methanol as an ionization accelerating solvent. The analytes were easily determined by comparing $[M+Cl]^-$ and $[M-H]^-$ [8,9]. Rat bile phosphatidylcholine (PC) was also characterized and determined using ESI-MS with chloroform [10]. Lipid extracts were infused on the condition of 3 μl/min in methanol–chloroform (2:1) and $[M+Cl]^-$ ions of PC were analyzed for the characterization. These methods which produced $[M+Cl]^-$ using chloroform, were kinds of soft ionization methods for HPLC-MS and useful for identifying molecular weights of heat-unstable compounds.

In our previous paper [11], soft ionization method adding accelerating solvents was developed for a heat-unstable antibiotic, FC/TA-891 and its active metabolite, FCE22101. FC/TA-891 was decomposed in the β-lactam ring by high source temperature of the APCI interface. For APCI-MS analysis of FC/TA-891 and FCE22101, chloroform and bromoform were investigated as accelerating solvents. Bromoform was employed for the first time in MS as an accelerating solvent. The HPLC–APCI-MS with chloroform and bromoform in the negative-ion mode, was applied to identify the molecular weights of FC/TA-891 and FCE22101. $[M+Br]^-$ ions were more stable and sensitive than $[M+Cl]^-$ ions in the APCI interface and could be easily identified from other ions. By this method using bromoform as an ionization accelerating solvent, the molecular weights of FC/TA-891 and FCE22101 could be identified clearly and easily.



penicillin (PC)	Abbeviation	R_1	R_2	R_3
benzylpenicillin	PCG	*a*	H	K
ampicillin	ABPC	*a*	NH_2	H
carbenicillin	CBPC	*a*	COOH	Na
sulbenicillin	SBPC	*a*	SO_3H	Na
piperacillin	PIPC	*a*	*d*	Na
aspoxicillin	ASPC	*b*	*e*	H
amoxicillin	AMPC	*b*	NH_2	H
ticarcillin	TIPC	*c*	COOH	H

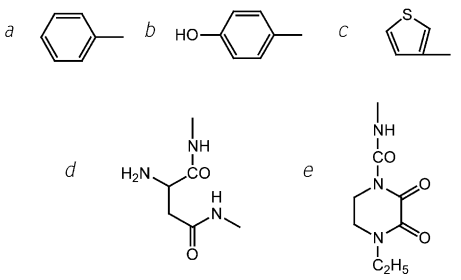


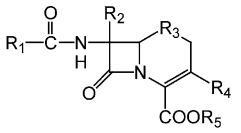
Fig. 1. Chemical structures of penicillins.

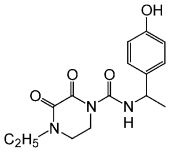
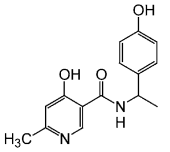
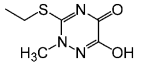
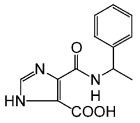
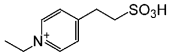
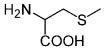
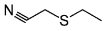
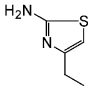
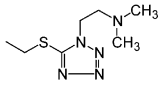
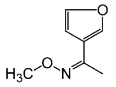
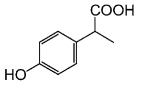
In this paper, the new method using bromoform in HPLC–APCI-MS under the negative-ion mode, was applied for β-lactam antibiotics which have similar chemical–physical properties, to identify their molecular weights and to investigate the scope or applicability of this method.

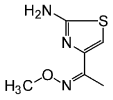
2. Experimental

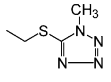
2.1. Materials and reagents

Penicillins; benzylpenicillin (PCG), ampicillin (ABPC), carbenicillin (CBPC), sulbenicillin (SBPC), piperacillin (PIPC), aspoxicillin (ASPC), amoxicillin (AMPC), ticarcillin (TIPC), and cephalosporins; cefotaxime (CTX), cefoperazone (CPZ), cefmenoxime (CMX), cefpiramide (CPM), ceftriaxone (CTRX), cefpimizole (CPIZ), cefminox (CMNX), ceftizoxime (CZX), cefoxitin (CFX), cefmetazole (CMZ), cefotiam (CTM),



cephalosporin (CEP)	Abbeviation	R_1	R_2	R_3	R_4	R_5
cefotaxime	CTX	*a*	H	S		Na
cefoperazone	CPZ		H	S	*b*	H
cefmenoxime	CMX	*a*	H	S	*b*	Na
cefpiramide	CPM		H	S	*b*	Na
ceftriaxone	CTRX	*a*	H	S		Na
cefpimizole	CPIZ		H	S		H
cefminox	CMNX		OCH_3	S	*b*	Na
ceftizoxime	CZX	*a*	H	S	H	H
cefoxitin	CFX		H	S	*c*	H
cefmetazole	CMZ		H	S	*b*	H
cefotiam	CTM		H	S		H
cefuroxime	CXM		H	S	*c*	H
latamoxef	LMOX		H	O	*b*	H

a 

b 

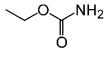
c 

Fig. 2. Chemical structures of cephalosporins.

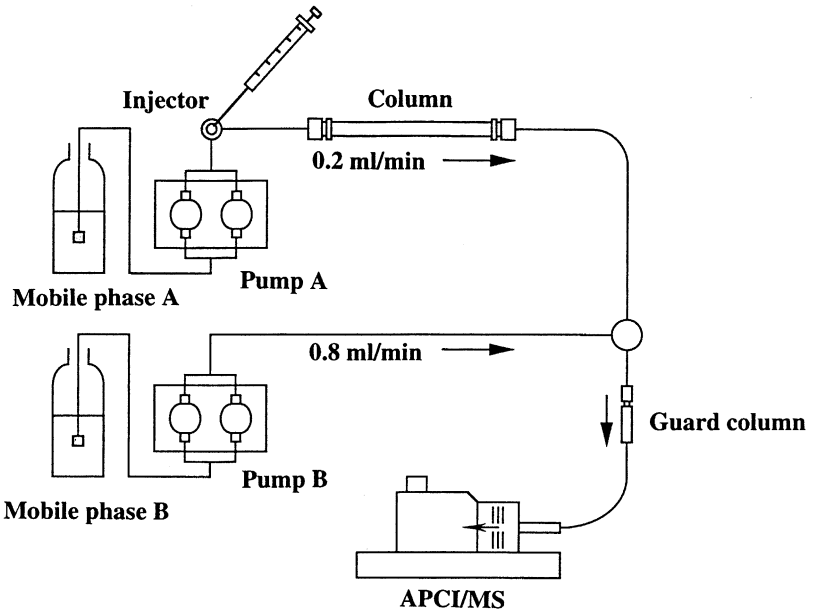


Fig. 3. Schematic illustration of the system of HPLC–APCI-MS.

cefuroxime (CXM), latamoxef (LOMX) were obtained through commercial sources. Structures of these samples are summarized in Figs. 1 and 2. Acetonitrile (CH_3CN), methanol (CH_3OH), acetone, tetrahydrofuran (THF), chloroform ($CHCl_3$) and 2-propanol (2-PrOH) were of HPLC grade, and were purchased from Katayama Chemical Industries (Osaka, Japan). Ammonium formate, ammonium acetate, formic acid and acetic acid were of guaranteed reagent grade and were purchased from Katayama Chemical Industries. Bromoform ($CHBr_3$) was of extra pure reagent grade and purchased from Tokyo Kasei Organic Chemicals (Tokyo, Japan). Trifluoroacetic acid (TFA) was of guaranteed reagent grade and purchased from Nacalai Tesque (Kyoto, Japan). Water was double distilled and purified by the Milli-Q water.

2.2. Apparatus

The HPLC–APCI-MS system used was a Model M-1000 (Hitachi, Tokyo, Japan) attached to a post-accelerator. This system was equipped with a quadrupole mass spectrometer. The two HPLC pumps used were Hitachi Model L-6200 with low-pressure gradient systems. Sample solutions were injected with a syringe via a Rheodyne Model 7161 loop injector. The loop volume was 20 μl. Separation was performed on an ODS column (TSK-gel 80TM, particle size 5 μm, 150 × 2.0 mm I.D.) (Tosoh, Tokyo, Japan). Mixing the mobile phases was performed on a guard column, LiChrospher 100 RP-18 endcapped (particle size 5 μm, 4.0 × 4.0 mm I.D.).

2.3. HPLC–APCI-MS conditions

The schematic illustration of the system is shown in Fig. 3. Flow rate of the mobile phase A was set at 0.2 ml/min and that of the mobile phase B was set at 0.8 ml/min. These two mobile phases were mixed with a trigonal cock and a guard column. The negative mode was used for MS detection. Each antibiotic was dissolved in water–acetnitrile (1:1, v/v). Injection amounts of all antibiotics were 20 μg.

For identifying molecular weights for antibiotics, water was used for the mobile phase A and CH_3CN was used for the mobile phase B without an accelerating solvent. Sample injection was performed without an HPLC column. The vapor-

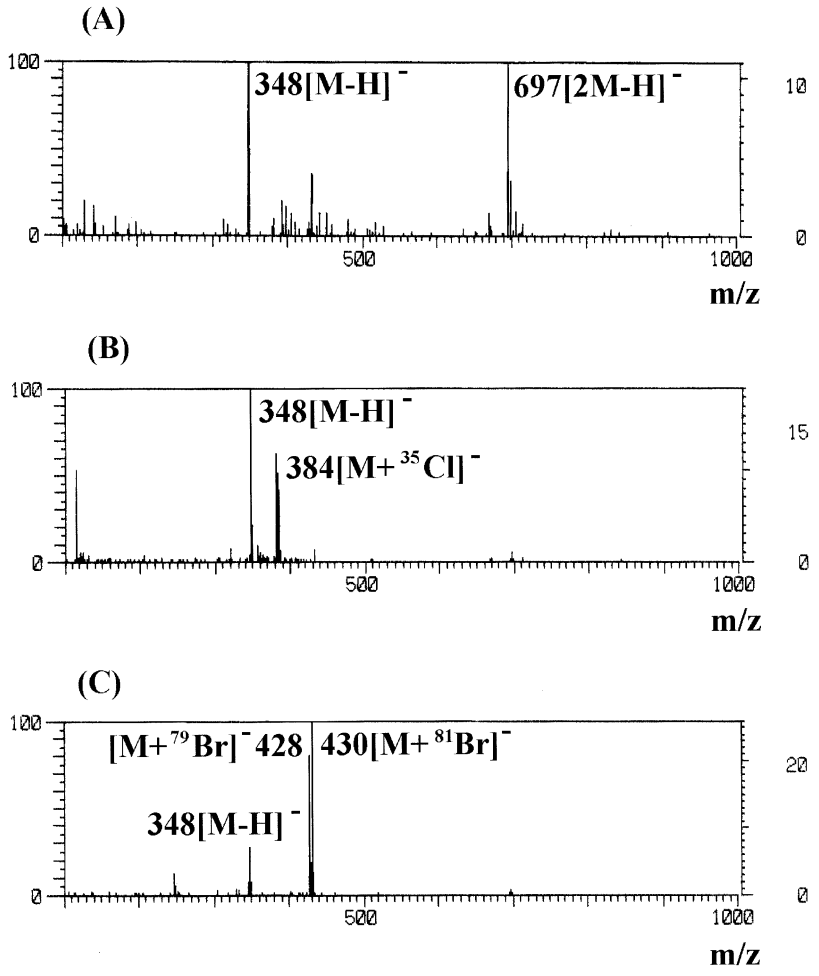


Fig. 4. Mass spectra of ampicillin (A) without accelerating solvents, (B) with $CHCl_3$ and (C) with $CHBr_3$.

izer, the desolvation region, the drift voltage, the focus voltage and the multiplier voltage were set at 200, 399 °C, −10, −110 V and 2.3 kV, respectively.

As a typical β-lactam antibiotic, ABPC was used for investigating the effects of the concentrations of the accelerating solvents and the species of the organic solvents in the mobile phase B, the vaporizer, the desolvation region, the drift voltage, the focus voltage and the multiplier voltage were set at the above-mentioned values. Water was used as the mobile phase A. For investigating the MS conditions, water was used as the mobile phase A, and a mixture of CH_3CN and an accelerating solvent (100:1, v/v) was used as the mobile phase B. Sample injections were performed without an HPLC column.

After optimizing the HPLC–APCI-MS conditions, a mixture of CH_3CN −1%(v/v) acetic acid (7:13, v/v) was used as mobile phase A for the separation of PCG, PIPC, CZX and CFX, and a mixture of CH_3CN and an accelerating solvent (100:1, v/v) was used as the mobile phase B. The vaporizer, the desolvation region, the drift voltage, the focus voltage and the multiplier voltage were set at the above-mentioned values.

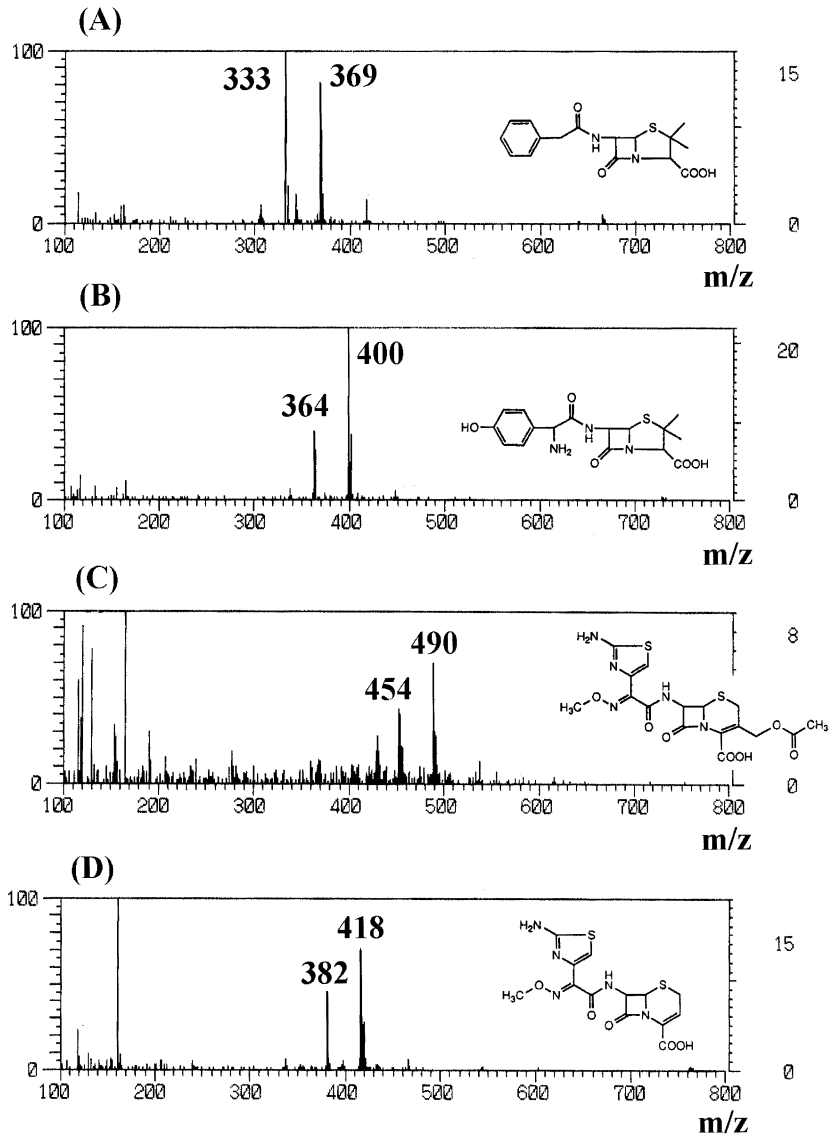


Fig. 5. Mass spectra of β-lactam antibiotics with $CHCl_3$ (A) benzylpenicillin, (B) amoxicillin, (C) cefotaxime, (D) ceftizoxime.

3. Results and discussion

3.1. HPLC–APCI-MS conditions

ABPC was used for investigating the effects of accelerating solvent concentrations and organic solvent species in the mobile phase B. First, the mobile phase conditions were optimized to obtain a strong ion intensity by changing various parameters. After changing $CHBr_3$ concentration in the mobile phase B, it was found that 1% (v/v) of $CHBr_3$ was enough to obtain the maximum ion intensity of the analytes. Next, the effects of organic solvent species in the mobile phase B on the ion intensity were investigated using CH_3CN, CH_3OH, 2-propanol, acetone and THF. Except for THF, there was no significant difference among the other four solvents. In using THF,

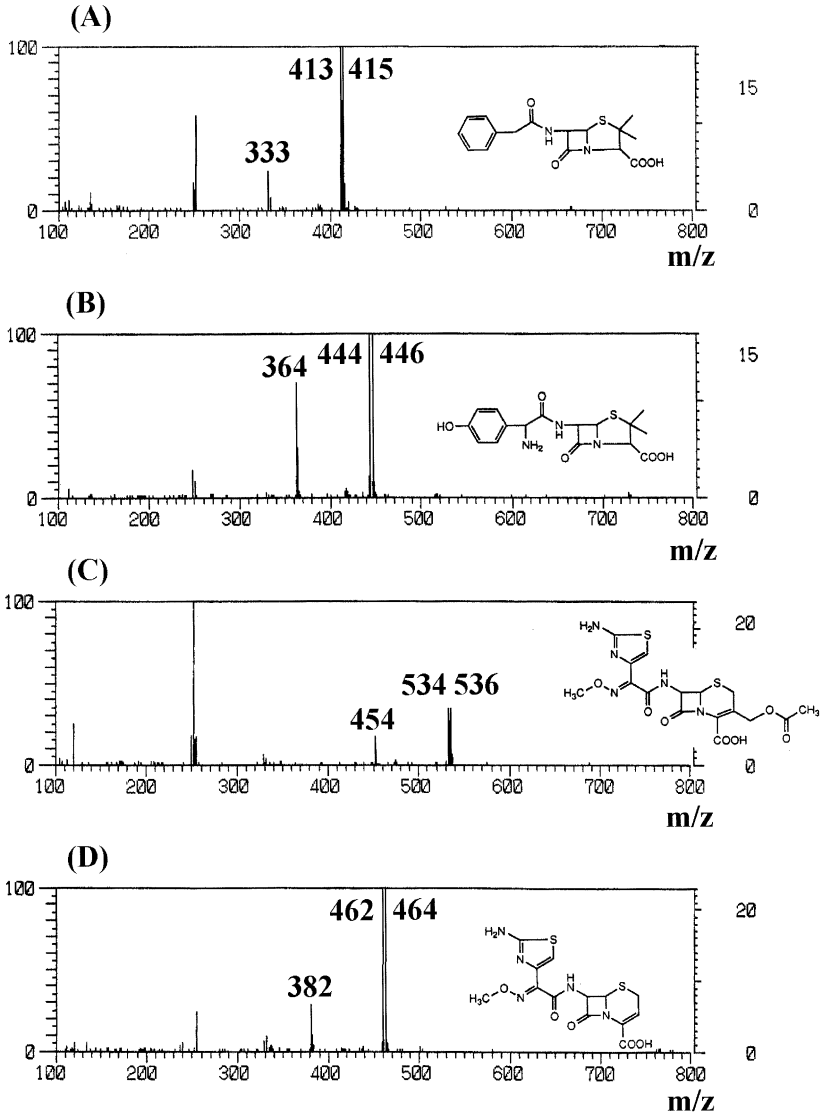


Fig. 6. Mass spectra of β-lactam antibiotics with $CHBr_3$ (A) benzylpenicillin, (B) amoxicillin, (C) cefotaxime, (D) ceftizoxime.

the adduct ion was not completely observed. It can be considered that an ion reaction between THF and CBr_3^- led to the suppression of the formation of an ion-molecule adduct.

The APCI-MS conditions, such as the temperature of the vaporizer (from 200 to 235 °C) and the drift voltage (from −30 to −10 V) were also optimized. The lower the temperature of the vaporizer or the higher the drift voltage, the higher was the intenstities of the ions. Consequently, a vaporizer temperature of 200 °C and a drift voltage of −10 V were selected as the optimum MS conditions. These conditions were the softest ionization conditions using HPLC–APCI-MS and were the same as those for FC/TA-891 and FCE22101 [11].

Without an ionization accelerating solvent, a strong $[M-H]^-$ ion and a strong dimmer ion of ABPC were obtained, as shown in Fig. 4A. In using $CHCl_3$, a strong $[M-H]^-$ ion, a medium

Table 1
The relative intensity ratios between the adducted ions and $[M-H]^-$

Common name	N.W. (free base)	Peak area ratio	
		$[M+^{35}Cl]^-/[M-H]^-$	$[M+^{79}Br]^-/[M-H]^-$
Penicillins			
Benzylpenicillin	334	0.81	4.08
Ampicillin	349	0.61	3.64
Carbenicillin	378	2.07	24.74
Sulbenicillin	414	N.D.	N.D.
Piperacillin	517	1.09	1.57
Aspoxicillin	493	2.11	7.35
Amoxicillin	365	3.44	3.31
Ticarcillin	384	1.65	15.55
Cephalosporins			
Cefotaxime	455	1.72	3.93
Cefoperazone	645	N.D.	N.D.
Cefmenoxime	511	N.D.	2.91
Cefpiramide	612	N.D.	N.D.
Ceftriaxone	554	N.D.	2.38
Cefpimizole	670	N.D.	N.D.
Cefminox	519	N.D.	1.94
Ceftizoxime	383	3.69	3.44
Cefoxitin	427	0.39	2.89
Cefmetazole	471	2.01	13.72
Cefotiam	525	N.D.	1.77
Cefuroxime	424	N.D.	3.00
Latamoxef	520	N.D.	N.D.

N.D.: Not detected.

$[M+^{35}Cl]^-$ and its isotopic ions were observed, as shown in Fig. 4B. The $[M+Cl]^-$ ions of ABPC did not give the stable isotope abundance ratio of 3:1. When ions are unstable, these phenomena are sometimes observed in HPLC–APCI-MS analysis. On the other hand, with $CHBr_3$, a strong $[M+^{79}Br]^-$ ion, a strong $[M+^{81}Br]^-$ ion and a weak $[M-H]^-$ ion of ABPC were observed, as shown in Fig. 4C. The intensities of $[M+Br]^-$ ions observed were in the ratio 1:1, indicating the stable isotope abundance ratio. Those intensity ratios corresponded accurately to the existing ratio of stable isotope abundance of the bromine. These results indicate that the $[M+Br]^-$ ions of ABPC are more stable and sensitive than the $[M+Cl]^-$ ions in the APCI interface, comparing with the $[M-H]^-$ ions. It could be easy to distinguish from other fragment ions and identify the molecular weight of ABPC by APCI-MS using $CHBr_3$.

3.2. Application to the identification of β-lactam antibiotics

Without an ionization accelerating solvent, the $[M-H]^-$ ion was not obtained from β-lactam antibiotics in Figs. 1 and 2, except for three of the penicillins; PCG, ABPC and AMPC. This means that PCG, ABPC and AMPC are relatively stable in the APCI-MS interface for the identification of the molecular weights. On the other hand, other β-lactam antibiotics are not stable to heat and the β-lactam ring probably decomposed in the interface. Therefore, a soft ionization method for HPLC–APCI-MS was required for these analytes.

The soft ionization methods of HPLC–APCI-MS [8,9,11], were applied to β-lactam antibiotics using $CHCl_3$ and $CHBr_3$ to demonstrate the advantage of ionization accelerating solvents. The results are shown in Figs. 5 and 6, and Table

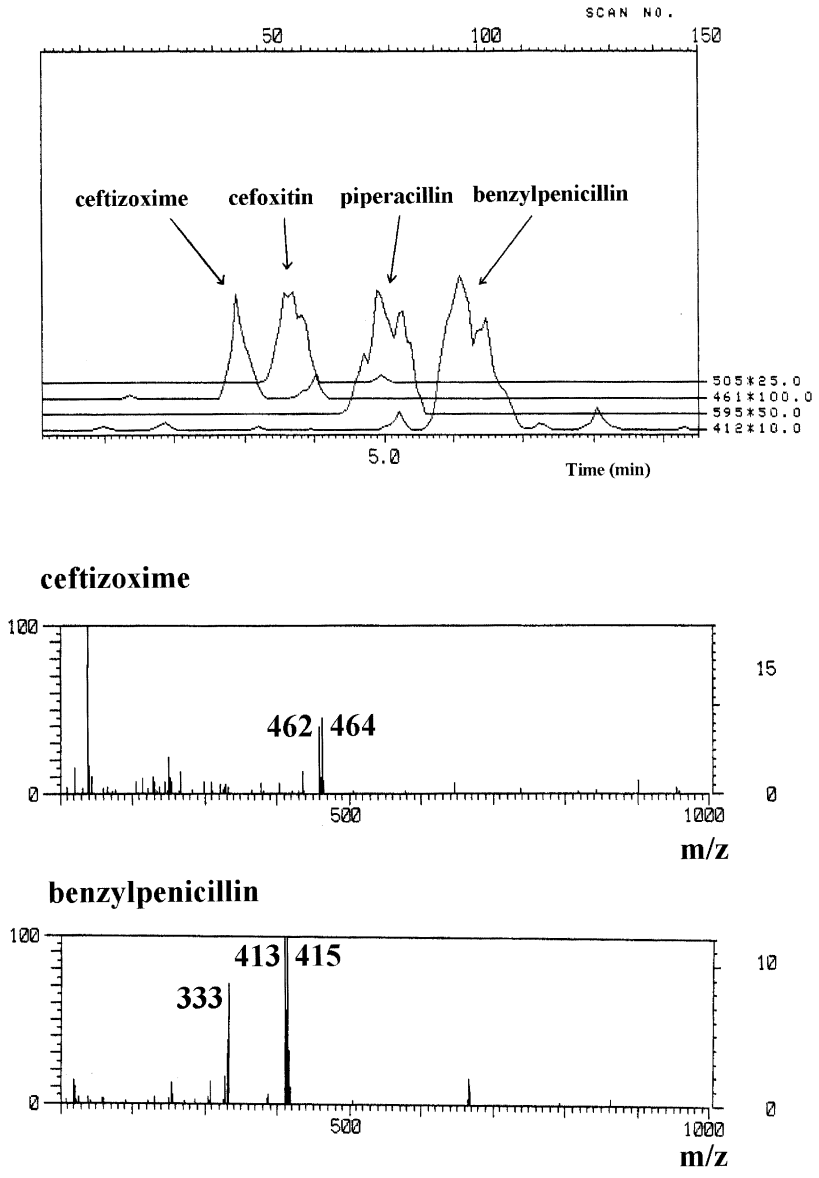


Fig. 7. Mass chromatograms and mass spectra of β-lactam antibiotics with $CHBr_3$.

1. The vaporizer, the desolvation region, the drift voltage, the focus voltage and the multiplier voltage were set at values mentioned in the experimental section.

In using $CHCl_3$, the $[M+^{35}Cl]^-$ ions of PCG, AMPC, CTX and CZX were observed at *m/z* 369, 400, 490 and 418, respectively, as shown in Fig. 5. The $[M+^{35}Cl]^-$ ions of penicillins except SBPC, and four compounds of cephalosporins were observed, but the $[M+Cl]^-$ ions for most of the β-lactam antibiotics did not give the stable isotope abundance ratio of 3:1. The relative intensity ratios between the $[M+^{35}Cl]^-$ ions and the $[M-H]^-$ ions were below 3.69, as shown in Table 1.

On the other hand, with $CHBr_3$, the $[M+^{79}Br]^-$ ions at *m/z* 413, 444, 534, 462 and the $[M+^{81}Br]^-$ ions at *m/z* 415, 446, 536, 464 were observed for PCG, AMPC, CTX and CZX, respectively, as

shown in Fig. 6. The $[M+Br]^-$ ions of penicillins except SBPC, and nine compounds of cephalosporins were observed, and the $[M+Br]^-$ ions for most of β-lactam antibiotics gave the stable isotope abundance ratio of 1:1. The relative intensity ratios between the $[M+^{79}Br]^-$ ions and the $[M-H]^-$ ions were below 24.74, as shown in Fig. 1.

The compounds which were not given the $[M+Cl]^-$ ions and the $[M+Br]^-$ ions, have relatively large molecular weights and negative groups within their chemical structures. Specifically, SBPC has a sulfuric acid group. Comparison with the $[M+Cl]^-$ ions, the $[M+Br]^-$ ions could be clearly distinguished from other ions because of the stable isotope abundance ratio of 1:1, and identified the molecular weights of β-lactam antibiotics. Judging from the number of measured $[M+Cl]^-$ ions or $[M+Br]^-$ ions, and the relative intensity ratios between those and the $[M-H]^-$ ions, we can conclude that the $[M+Br]^-$ ions were more stable and sensitive than the $[M+Cl]^-$ ions because of their electronegativity.

3.3. Application to the separation of β-lactam antibiotics

Two penicillins and two cephalosporins were applied to an HPLC separation. After optimizing the HPLC–APCI-MS conditions, a mixture of CH_3CN −1%(v/v) acetic acid (7:13, v/v) was used as the mobile phase A for the separation of PCG, PIPC, CZX, CFX, and a mixture of CH_3CN–$CHBr_3$ (100:1, v/v) was used as the mobile phase B. The vaporizer, the desolvation region, the drift voltage, the focus voltage and the multiplier voltage were set at the same values of above-mentioned. As shown in Fig. 7, PCG, PIPC, CZX and CFX were completely separated by HPLC under the above-mentioned conditions. Strong $[M+^{79}Br]^-$ and $[M+^{81}Br]^-$ ions of CZX and PCG were observed. The intensities of these ions were in the ratio about 1:1, indicating a stable isotope abundance ratio. These results were the same as in the flow injection mode. Consequently, the method using $CHBr_3$ could be applied to the identification of β-lactam antibiotics.

4. Conclusions

The HPLC–APCI-MS method described in this paper using $CHBr_3$ as an ionization accelerating solvent, allowed the observation of the adduct ions for β-lactam antibiotics. The $[M+Br]^-$ ions were more stable and sensitive than the $[M+Cl]^-$ ions in the APCI interface and could be identified easily from other fragment ions. This soft ionization method enabled clearly and easily to identify the molecular weights of penicillins and cephalosporins. This method will be probably applied to other interfaces of HPLC-MS.

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