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Capillary high-performance liquid chromatography–fast atom bombardment mass spectrometry of 24 cephem antibiotics

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Abstract

Using capillary high-performance liquid chromatography (HPLC)–fast atom bombardment (FAB) mass spectrometry (MS), both positive and negative FAB mass spectra of 24 cephem antibiotics with diethanolamine (DEA) and glycerol (GLY) as matrices are presented. In the positive mode, an internal quasi-molecular peak together with relatively abundant fragment peaks were obtained from all 24 drugs with both matrices, though DEA provided more information on molecular mass of a compound than did GLY for some drugs. In the negative mode, the background was generally lower than that in the positive, but neither the quasi-molecular nor molecular peak was detected in several drugs with either matrix. The drugs were isolated from serum samples using an octadecyl reversed-phase cartridge; recoveries were generally over 60%. With this isolation and the capillary HPLC–FAB-MS in the positive mode, ceftriaxone and cefazolin, two of the most popular cephem antibiotics, were successfully identified in 0.5 ml of sera obtained from a clinical or an autopsy case.

Keywords: Cephem antibiotics

1. Introduction

Cephem antibiotics are widely used in the treatment of bacterial infections because they are highly antimicrobial against a large number of both Gram-positive and Gram-negative organisms. However, in forensic practice, fatal cases due to shock following the use of cephem antibiotics are still occasionally encountered [1]. Cephem antibiotics are not suitable for analysis by conventional gas chromatography (GC)–mass spectrometry (MS) in their underivatised forms owing to their polar, non-volatile and thermolabile properties. For MS analyses of such compounds, fast atom bombardment (FAB) is one of the most reliable techniques. FAB-MS analyses of cephalosporins, representative cephem antibiotics, have been reported for standard compounds in chemical and pharmacological laboratories [2,3]. However, these studies utilized direct inlet methods unsuitable for analyses of drugs in biological samples encountered in clinical and forensic laboratories. For such analysis, the combination of high-performance liquid chromatography (HPLC) with MS, allowing on-line mass analysis of a compound, is the most desirable [4,5].

Recently, we reported a detailed procedure for the detection of cefaclor, one of the most popular cephem antibiotics, in human serum by capillary HPLC–FAB-MS [6]. A special column-switching device for HPLC injection and a capillary HPLC

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column resulted in much higher sensitivity, enabling mass spectral identification of cefaclor in human serum after oral administration of a small dose of the drug [6].

In this paper, using a minor modification of our previous HPLC–MS system [5,6], we present the FAB mass spectra of 24 cephem antibiotics in both positive and negative modes with diethanolamine (DEA) and glycerol (GLY) as matrices. With the present HPLC–MS system, we were able to successfully identify two drugs, ceftriaxone and cefazolin, in human sera using octadecyl reversed-phase cartridge extraction, after intravenous dosing.

2. Experimental

2.1. Chemicals

Molecular structures and masses of 24 cephem antibiotics used in the present study are shown in Fig. 1, in which the order of the drugs are arranged on the basis of common moieties in the structures. The pure powder of the cephem antibiotics were kindly supplied by the following Japanese pharmaceutical companies: I, II, XVI and XVII by Takeda Chem. Ind. Co., Osaka; III, XVIII and XIX by Fujisawa Pharmaceutical Co., Osaka; IV, and VII, VIII, IX, X, XI, XXIII and XXIV by Shionogi and Co., Osaka; V and XX by Toyama Chem. Ind., Toyama; VI and XXII by Yamanouchi Pharmaceutical Co., Tokyo; XII and XIII by Tanabe Pharmaceutical Co., Tokyo; XIV by Hoechst Japan Co., Tokyo; XV by Roche Japan Co., Tokyo; XXI by Sankyo Co., Tokyo. Standard solutions were prepared by dissolving each compound in deionised water at varying concentrations from 0.1 to 50 μg/ml. Bond-Elut C_{18} cartridges (200 mg/3 ml) were purchased from Varian (Harbor City, CA, USA), and the other chemicals used were of the highest purity commercially available.

2.2. Capillary high-performance liquid chromatographic system

The same system as described previously [5,6] was used, except that the capillary columns and mobile phases were modified as follows. The separation capillary column (15 cm × 0.5 mm I.D.), packed with 5-μm particle size Develosil PhA packing material, and the concentration column (3 cm × 0.5 mm I.D.), packed with 10 μm of the Develosil PhA packing material, were purchased from Nomura Chemical Co. (Seto, Aichi, Japan). Phenethyl groups are bonded to silica groups in the Develosil PhA. The mobile phase for the separation capillary column consisted of deionised water–methanol–acetic acid–GLY (59:40:0.5:0.5, v.v) or deionised water–methanol–acetic acid–DEA (57:40:2.5:0.5, v/v); pH values of both mobile phases were approximately 3, and the flow-rate for the separation column was 4 μl/min. Another mobile phase, for sending sample fluid to the concentration column at a rate of 0.05 ml/min, consisted of 0.01 *M* ammonium acetate solution–GLY or DEA (99.5:0.5, v/v); these mixtures were adjusted to pH 5 with acetic acid. In this system, the capillary column (0.5 mm I.D.) enabled the introduction of the entire effluent to the frit interface for FAB-MS without any splitting; a special column-switching device for injection and concentration enabled injection of volumes as large as 500 μl [5,6]. In this HPLC system, some cephalosporins could be determined with a ten-fold increase in sensitivity [7] over a conventional HPLC procedure [8].

2.3. Interface for high-performance liquid chromatography–fast atom bombardment mass spectrometry

The interface between the HPLC system and the MS instrument was a frit made of a porous stainless filter and fused silica tubing (0.05 mm I.D.); the details were described previously [5].

2.4. Fast atom bombardment mass spectrometric conditions

The same conditions as described previously [5,6] were used except that determination was made in both positive and negative modes in this study.

2.5. Purification of clinical and autopsy blood

For treatment of a urinary infection, a 32-year-old male in-patient received an intravenous injection of

(H3CO) H (O) S
R1—CONH— [cephem nucleus] —X—R2
O N
R3

	Compound (M.W.)	R_1	X	R_2	R_3
I.	Cefsulodin[a] (532)	SO_3H, C, H	$-CH_2-$	^{+}N, $CONH_2$	$-COO^-$
II.	Cefotiam[b] (525)	H_2N, N, S, CH_2-	$-CH_2-$	-S, N, N, N, N, $CH_2CH_2N(CH_3)_2$	$-COOH$
III.	Cefazolin[a] (454)	N=N, N, N-CH_2-	$-CH_2-$	-S, N, N, S, CH_3	$-COOH$
IV.	Cefamandole[a] (462)	OH, C, H	$-CH_2-$	-S, N, N, N, N, CH_3	$-COOH$
V.	Cefoperazone[a] (645)	HO, H, C, NHCO-N, O, O, N-C_2H_5	$-CH_2-$	-S, N, N, N, N, CH_3	$-COOH$
VI.	Cefpiramide[a] (612)	HO, H, C, NH, CO, N, H_3C, OH	$-CH_2-$	-S, N, N, N, N, CH_3	$-COOH$
VII.	Cephaloridine (415)	S, CH_2-	$-CH_2-$	^{+}N	$-COO^-$
VIII.	Cephalotin[a] (396)	S, CH_2-	$-CH_2-$	$-OCOCH_3$	$-COOH$
IX.	Cephaloglycin (405)	NH_2, C, H	$-CH_2-$	$-OCOCH_3$	$-COOH$
X.	Cefaclor (367)	NH_2, C, H	$-Cl$	-	$-COOH$
XI.	Cephalexin (347)	NH_2, C, H	$-CH_3$	-	$-COOH$
XII.	Cefuroxime[a] (424)	O, C-, $NOCH_3$	$-CH_2-$	$-OCONH_2$	$-COOH$
XIII.	Ceftazidime (546)	H_2N, N, S, C-, N, O-C-COOH, CH_3, CH_3	$-CH_2-$	^{+}N	$-COO^-$

Fig. 1. Chemical structures of cephem antibiotics in the present study: (a) compounds were used as sodium salt; (b) used as dihydrochloride salt; (c) used as hemihydrochloride salt.

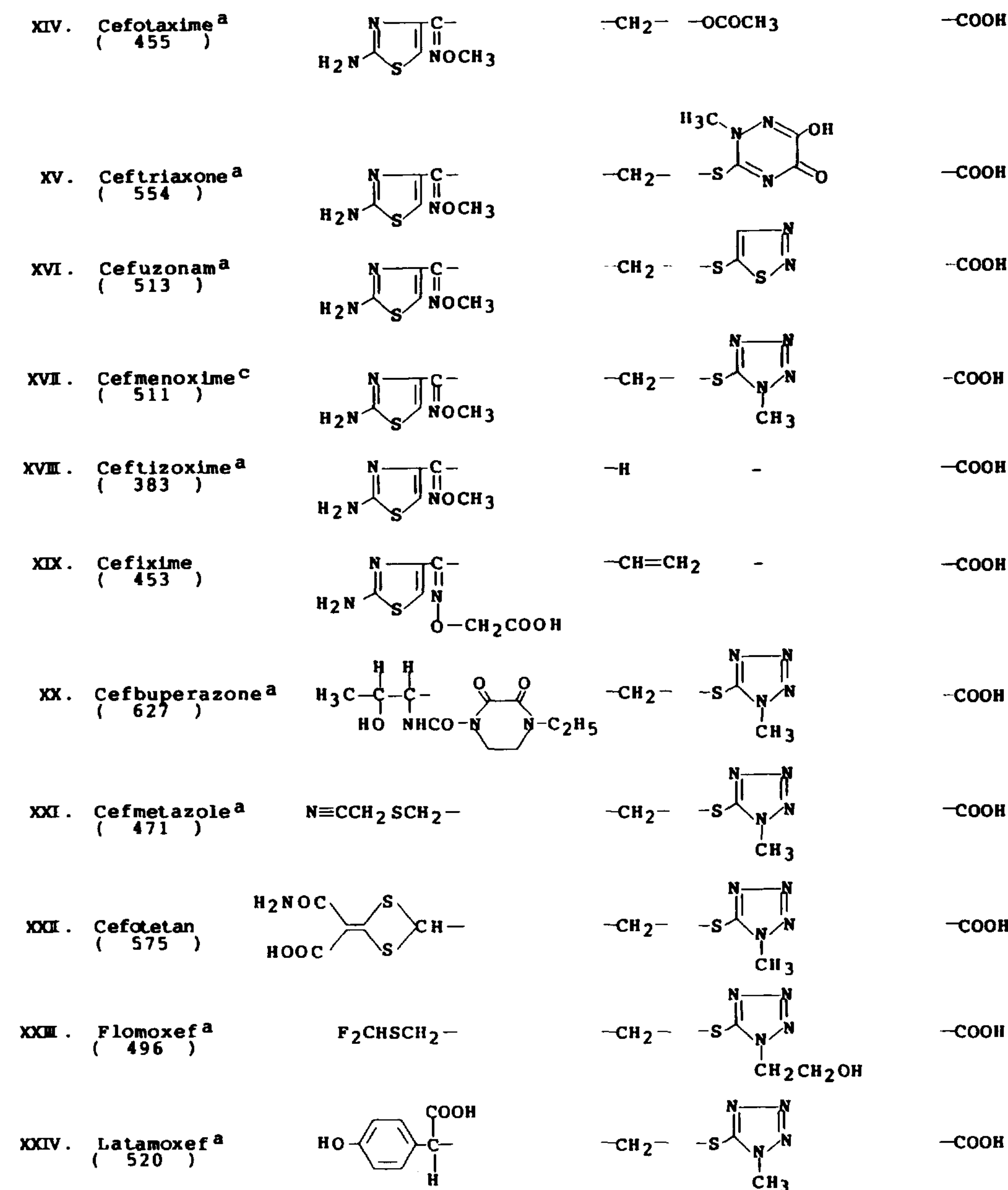

Fig. 1. (*cont.*)

1 g of ceftriaxone sodium (XV) dissolved in 100 ml of saline. Informed consent was obtained from the subject, and 2 ml of blood were taken from him 4 h after the injection.

For treatment of a respiratory infection, a female out-patient of 60 years of age was injected intravenously with 1 g of cefazolin sodium (III) dissolved in 100 ml of saline. She fell into shock 15 min after the injection and various emergency procedures were employed. Nevertheless, the patient died 90 min after the injection. An autopsy was performed approximately 24 h after death. From the autopsy, the cause

of death was thought to be shock following the intravenous injection of cefazolin sodium. Blood was sampled at the time of the autopsy.

Each serum was separated by centrifugation (7000 *g*, 15 min), and the drugs were isolated using Bond-Elut C_{18} cartridges according to the manufacturer's manual with a minor modification, the details of which were described in our previous paper [6]. Only 0.5 ml of serum was used in the isolation procedure. The recoveries of XV and III, which had been added to blank sera, were 63.4±7.2% and 92.5±5.0% ($n = 4$) for the Bond-Elut C_{18} isolation procedure, respectively. Those of other drugs were over 70% except for I (about 40%).

3. Results

3.1. Positive FAB mass spectra with GLY

Table 1 shows the principal ions in a positive FAB mass spectra of 24 cephem antibiotics with GLY. Under the acidic conditions used, all 15 sodium salt compounds formed free acids due to the replacement of a sodium ion with a proton, and the free acids are expressed as their molecules (M) in this study; quasi-molecular ions were detected at m/z M+H but not at m/z M+Na. Similar to the above, dihydrochloride or hemihydrochloride salt compounds formed free acids (M), and quasi-molecular ions were detected at m/z M+H. Various intensities of the $[M+H]^+$ quasi-molecular ions appeared for all 24 compounds; they were low (3–5%) for six compounds (V, VI, VIII, XII, XV and XX) whereas the quasi-molecular ions constituted base peaks for two compounds (XVIII and XXIII). In addition, small cations at m/z M+H+92, which were considered to be quasi-molecular adduct ions with GLY, were observed in ten compounds.

Fig. 2 shows the principal fragmentation pathways proposed in this study. Cations due to the liberation of the side chains (R_1) appeared at m/z R_1 for most compounds and constituted base peaks for nine compounds (IV, VII–XII, XXI and XXII). Cations derived from decomposition products of the liberated side chains (R_1) occurred for 13 compounds and gave base peaks for eight compounds (V, VI, XIV–XVII, XIX and XX). Cations due to the liberation of another side chain (R_2) also appeared at m/z R_2+H or R_2+2H for 12 compounds (the former R_2 was always a pyridinyl ring) and gave base peaks for four compounds (I–III and XIII). Thus, the fragment ions derived from liberated R_1 or R_2, or the decomposition products of R_1, constituted base peaks for all compounds except three (XVIII, XXIII and XXIV).

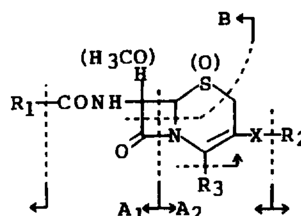

Fig. 2. Fragmentation pathways of cephem antibiotics proposed in this study.

Other cations caused by the liberation of R_2 were observed at m/z M–R_2 or M–R_2+H for most compounds (the latter R_2 was always a pyridinyl ring), though their intensities varied. In addition, cations due to a cleavage of the lactam ring were observed at m/z A_1+H for 18 compounds and at m/z A_2+H for 15 compounds though their intensities were relatively low. Other fragment ions with intensities of 15% or more are listed in the column for "Others" in Table 1. Cations due to transection over both lactam and thiazine (oxadine) rings were observed at m/z B+2H for some compounds, but the intensities were less than 15% (data not shown).

The detection limits of the mass spectral measurements were 10–50 ng on the column for IX–XII, 50–200 ng for IV–VIII, XIV, XXI and XXII, 200–1000 ng for III, XIII, XVI–XX, XXIII and XXIV, and 1–2.5 μg for I, II and XV.

3.2. Negative FAB mass spectra with GLY

Table 2 shows the principal ions in negative FAB mass spectra of the 24 antibiotics with GLY. No peaks with sodium metal or hydrochlorides were observed. Various intensities of the $[M-H]^-$ quasi-molecular ions appeared for 20 compounds, and slightly higher intensities of molecular anions than those of $[M-H]^-$ also appeared for two compounds having a pyridinyl ring (VII and XIII). Neither

Table 1
Principal ions[a] in positive FAB mass spectra of 24 cephem antibiotics with GLY

Compound no.	m/z (% I)							
	M+H	M+H+GLY	A_1+H	A_2+H	R_1	R_2+2H	M-R_2	Others[b]
I	533 (12)	625 (3)	nd[c]	nd	nd	123 (100)[d]	411 (3)[e]	80 (60):R_2–$CONH_2$+2H, 91 (35):R_1–SO_3H+H, 188 (30)
II	526 (20)	618 (3)	198 (10)	nd	113 (30)	174 (100)	353 (8)	72 (45):$CH_2CH_2N(CH_3)_2$, 114 (40), 58 (35):$CH_2N(CH_3)_2$, 141 (15)
III	455 (19)	nd	nd	nd	nd	133 (100)	323 (15)	112 (23), 156 (22), 100 (17)
IV	463 (19)	nd	192 (15)	272 (7)	107 (100)	117 (26)	347 (47)	79 (63):R_1–CHOH+2H, 158 (62), 105 (53), 141 (40), 139 (38), 91 (27)
V	646 (3)	nd	nd	nd	290 (7)	nd	530 (3)	143 (100):R_1–$CONHCHC_6H_4OH$+2H, 148 (40), 70 (16), 122 (15)
VI	613 (5)	nd	nd	nd	257 (22)	nd	nd	136 (100):R_1–$NHCHC_6H_4OH$, 122 (58), 109 (22), 153 (17)
VII	416 (52)	508 (3)	182 (4)	235 (3)	97 (100)	80 (90)[d]	337 (24)[e]	145 (27), 152 (17), 142 (16)
VIII	397 (3)	nd	182 (7)	216 (6)	97 (100)	nd	337 (16)	156 (20)
IX	406 (22)	498 (5)	191 (4)	216 (3)	106 (100)	nd	346 (22)	118 (26), 158 (16)
X	368 (65)	460 (14)	191 (11)	178 (9)	106 (100)	–	–	118 (24), 174 (15)
XI	348 (68)	440 (16)	191 (6)	158 (30)	106 (100)	–	–	118 (22)
XII	425 (5)	nd	209 (30)	217 (10)	124 (100)	nd	364 (70)	336 (40), 109 (37), 156 (35), 94 (20):R_1–OCH_3+H, 139 (20), 179 (20)
XIII	547 (42)	639 (4)	313 (3)	235 (5)	nd	80 (100)[d]	468 (23)[e]	126 (90):R_1–$OC(CH_3)_2COOH$+H, 142 (62), 145 (30), 218 (25), 112 (16)
XIV	456 (60)	nd	241 (15)	216 (3)	156 (70)	nd	396 (5)	126 (100):R_1–OCH_3+H, 141 (20), 182 (20), 211 (20)
XV	555 (5)	nd	241 (8)	nd	156 (50)	160 (23)	396 (8)	126 (100):R_1–OCH_3+H, 141 (25), 211 (15)
XVI	514 (56)	nd	241 (6)	nd	156 (40)	nd	396 (4)	126 (100):R_1–OCH_3+H, 141 (15), 398 (15)
XVII	512 (16)	nd	241 (8)	nd	156 (70)	117 (12)	396 (10)	126 (100):R_1–OCH_3+H, 141 (22), 182 (20), 211 (15)
XVIII	384 (100)	476 (9)	241 (8)	144 (11)	156 (20)	–	–	126 (78):R_1–OCH_3+H
XIX	454 (37)	546 (5)	285 (5)	170 (10)	200 (7)	–	–	126 (100):R_1–OCH_2COOH+H
XX	628 (3)	nd	nd	nd	242 (3)	nd	512 (3)	143 (100):R_1–$CONHCHCH(CH_3)OH$+2H, 70 (20)
XXI	472 (60)	nd	201 (5)	272 (10)	86 (100)	117 (90)	356 (45)	215 (90), 104 (60), 112 (60), 156 (60), 328 (55), 139 (40), 171 (40), 197 (20), 284 (15)
XXII	576 (10)	nd	nd	272 (13)	190 (100)	117 (50)	460 (5)	146 (90), 112 (70), 156 (70), 85 (65), 218 (60), 102 (50), 171 (50), 139 (40), 215 (40), 432 (20)
XXIII	497 (100)	589 (3)	212 (18)	286 (5)	97 (83)	147 (73)	351 (30)	199 (64), 323 (50), 88 (40), 137 (30), 155 (28), 112 (22)
XXIV	521 (30)	nd	266 (20)	256 (7)	151 (60)	117 (95)	405 (18)	199 (100), 377 (40), 88 (30)

[a] Arranged mainly according to the proposed fragmentation pathways shown in Fig. 2.
[b] Isotopic peaks and peaks of intensities less than 15% were omitted.
[c] Not detectable (intensity less than 3%).
[d] R_2 (pyridinyl ring)+H.
[e] M–R_2 (pyridinyl ring)+H.

Table 2
Principal ions[a] in negative FAB mass spectra of 24 cephem antibiotics with GLY

Compound no.	m/z (% I)						
	M–H	M–H+GLY	A_1–H	B	R_2	M–H–CO_2	Others[b]
I	nd[c]	nd	nd	272 (2)	nd	nd	80 (100):SO_3, 170 (22)
II	524 (8)	nd	196 (10)	nd	172 (100)	nd	
III	nd	nd	166 (7)	nd	131 (80)	nd	58 (100), 69 (72), 98 (15)
IV	461 (4)	nd	190 (10)	208 (10)	115 (100)	nd	
V	644 (2)	nd	nd	nd	115 (100)	nd	
VI	611 (6)	703 (2)	340 (5)	nd	115 (100)	nd	
VII	414 (7)	506 (7)	180 (100)	198 (72)	nd	370 (7)	167 (45), 23 (32), 156 (30), 100 (30), 155 (28), 74 (25), 139 (20), 112 (20), 292 (17)
	415 (10)[d]						
VIII	395 (90)	487 (35)	180 (100)	198 (66)	nd	351 (25)	197 (56):M–H–B, 156 (38), 291 (38), 167 (35), 100 (27), 123 (27), 112 (21), 74 (17), 224 (15), 335 (15)
IX	404 (50)	496 (20)	189 (100)	207 (41)	nd	360 (17)	344 (40), 156 (35), 197 (35):M–H–B, 100 (32), 300 (30), 233 (20)
X	366 (47)	458 (17)	189 (90)	207 (100)	–	322 (87)	233 (85), 153 (82), 159 (55):M–H–B, 176 (52):A_2–H, 286 (48), 127 (45), 100 (37), 733 (18):2M–H
XI	346 (83)	438 (14)	189 (78)	207 (85)	–	302 (52)	139 (100):M–H–B, 100 (48), 135 (38), 156 (38):A_2–H, 112 (30), 173 (23), 233 (15)
XII	423 (42)	515 (3)	207 (87)	225 (36)	60 (100)	379 (5)	100 (48), 153 (22), 82 (22), 155 (20), 193 (20), 318 (20), 198 (17):M–H–B, 336 (15)
XIII	545 (5)	637 (4)	311 (20)	nd	nd	nd	103 (100):$OC(CH_3)_2COOH$, 124 (40):R_1–$OC(CH_3)_2COOH$–H, 100 (15)
	546 (6)[d]						
XIV	454 (76)	546 (18)	239 (50)	257 (10)	nd	410 (10)	124 (100):R_1–OCH_3–H, 82 (48), 197 (45):M–H–B, 100 (34), 350 (28), 394 (27), 153 (22), 167 (20), 209 (20)
XV	nd	nd	239 (5)	nd	158 (100)	nd	58 (18), 144 (17)
XVI	512 (4)	nd	239 (5)	nd	117 (100)	nd	124 (18):R_1–OCH_3–H, 82 (16)
XVII	510 (8)	602 (3)	239 (4)	nd	115 (100)	nd	58 (38)
XVIII	382 (52)	474 (26)	239 (30)	257 (12)	–	338 (15)	124 (100):R_1–OCH_3–H, 125 (71):M–H–B, 82 (53), 100 (27)
XIX	452 (20)	nd	283 (10)	301 (3)	–	408 (3)	75 (100):OCH_2COOH, 82 (50), 124 (80):R_1–OCH_2COOH–H, 209 (20), 100 (20)
XX	626 (2)	nd	nd	nd	115 (100)	nd	
XXI	470 (10)	562 (4)	nd	nd	115 (100)	nd	72 (15):$NCCH_2S$
XXII	574 (3)	nd	nd	nd	115 (100)	nd	100 (62), 146 (18), 158 (18), 176 (17)
XXIII	495 (15)	nd	nd	nd	145 (100)	451 (5)	83 (50):F_2CHS
XXIV	nd	nd	nd	nd	115 (100)	nd	

[a] Arranged mainly according to the proposed fragmentation pathways shown in Fig. 2.
[b] Isotopic peaks and peaks of intensities less than 15% were omitted.
[c] Not detectable (intensity less than 2%).
[d] M.

quasi-molecular nor molecular anions were detected in four compounds (I, III, XV and XXIV). Quasi-molecular adduct anions with matrix were observed at *m/z* M–H+GLY for 13 compounds, although the intensities were relatively low.

Anions due to the liberation of the side chains (R_2) appeared as intense peaks in 14 compounds and constituted base peaks for 13 compounds (II, IV–VI, XII, XV–XVII and XX–XXIV). Anions due to a cleavage of the lactam ring occurred with various intensities at *m/z* A_1–H for most compounds and constituted base peaks for three compounds (VII–IX). Other anions due to the cleavage were observed at *m/z* A_2–H, but this was only for a few compounds. Various intensities of anions due to transection over both the lactam and thiazine (oxadine) ring appeared at *m/z* B for 11 compounds, and one of them gave a base peak (X). Other anions due to the transection were observed at *m/z* M–H–B with various intensities for seven compounds, and one of them constituted a base peak (XI). In addition, anions caused by the decarboxylation from $[M-H]^-$, which were specific to the negative mode, occurred in ten compounds.

Sensitivity of the mass spectral measurement in the negative mode was almost comparable with the compounds having intense peaks of R_2 anions, but was one order of magnitude lower for other compounds, as compared with that in the positive mode. The number of total ions in the negative mode was generally much less than that in the positive mode, and the background in the negative spectra was also less than that in the positive. The cut-off value for detection of peaks in the negative mode (2%) was slightly lower than that in the positive mode (3%).

3.3. Positive FAB mass spectra with DEA

Table 3 shows the principal ions in their positive mass spectra with DEA. The proposed fragmentation pathways in this mode were very similar to those in the positive mode with GLY, except that many compounds gave fragment adduct ions with matrix. $[M+H+DEA-R_2]^+$ ions were especially often observed. All 24 compounds gave quasi-molecular ions $[M+H]^+$ together with their adduct ions with matrix $[M+H+DEA]^+$; either $[M+H]^+$ or $[M+H+DEA]^+$ showed over 7% intensities for all compounds and constituted base peaks for 16 compounds. In particular, the quasi-molecular adduct ions with matrix, which were generally small for GLY, gave base peaks for 13 compounds. Another quasi-molecular adduct ions at *m/z* M+H+2DEA also appeared for most compounds.

The detection limits of the mass spectral measurements were almost comparable with those in the positive mode with GLY, except for three compounds (IX–XI) where the liberated R_1 cation at *m/z* 106 gave the base peak with GLY. With DEA, the R_1 cation could not be detected because the protonated matrix ion $[DEA+H]^+$ at the same *m/z* co-existed in abundance; the sensitivity for the three compounds with DEA was one order of magnitude lower than that with GLY.

3.4. Negative FAB mass spectra with DEA

Table 4 shows the principal ions in their negative spectra with DEA. The formation of quasi-molecular adduct anions with DEA occurred much less often than that of cations in the positive mode, and low intensities of the adduct anions were observed at *m/z* M–H+DEA for nine compounds. Quasi-molecular, molecular and fragment anions in this mode were almost similar to those in the negative mode with GLY.

Sensitivities of the mass spectral measurements were almost comparable with those in the negative mode with GLY for all compounds.

3.5. Retention time on mass chromatograms of 24 cephem antibiotics

Table 5 shows the retention time on mass chromatograms of 24 cephem antibiotics under the same conditions as described above. All drugs could be detected within 40 min, though they could not be clearly separated from each other on the mass chromatograms, especially in the mobile phase with DEA.

3.6. HPLC–FAB-MS analysis of clinical or autopsy samples

For ceftriaxone sodium, measurements were made in the positive mode with DEA because an intense

Table 3
Principal ions[a] in positive FAB mass spectra of 24 cephem antibiotics with DEA

Compound no.	m/z (% I)									
	M+H	M+H+DEA	M+H+2DEA	A_1+H	A_2+H	R_1	R_2+2H	M–R_2	M+H+DEA–R_2	Others[b]
I	533 (10)	638 (3)	nd[c]	nd	278 (4)	nd	123 (100)[d]	411 (3)[e]	517 (5)[f]	91 (52), 188 (28), 80 (23)
II	526 (43)	631 (20)	736 (5)	198 (25)	nd	113 (95)	174 (90)	353 (35)	459 (20)	58 (100), 72 (90), 141 (90), 114 (42), 279 (22)
III	455 (20)	560 (100)	665 (12)	nd	nd	nd	133 (60)	323 (15)	429 (25)	156 (20)
IV	463 (7)	568 (100)	673 (3)	nd	nd	nd	117 (14)	347 (22)	453 (12)	185 (33), 79 (25), 141 (25), 158 (22), 91 (20)
V	646 (3)	751 (7)	–	nd	nd	290 (10)	nd	530 (4)	nd	143 (100), 148 (30)
VI	613 (7)	718 (5)	–	nd	nd	257 (80)	nd	nd	nd	136 (100), 153 (78), 122 (75), 109 (37), 285 (20)
VII	416 (100)	521 (20)	nd	182 (4)	235 (3)	97 (60)	80 (95)[d]	337 (22)[e]	443 (8)[f]	185 (28), 145 (18), 152 (16), 142 (15)
VIII	397 (3)	502 (100)	607 (17)	182 (7)	216 (4)	97 (95)	nd	337 (10)	443 (7)	
IX	406 (18)	511 (100)	616 (7)	191 (3)	216 (11)	nd	nd	346 (40)	452 (7)	91 (40):R_1–NH_2+H, 158 (35), 79 (27), 174 (22), 118 (16)
X	368 (34)	473 (100)	578 (28)	191 (10)	178 (6)	nd	–	–	–	118 (34), 247 (29), 91 (18):R_1–NH_2+H, 174 (16)
XI	348 (28)	453 (100)	558 (9)	191 (4)	158 (46)	nd	–	–	–	118 (45), 91 (35):R_1–NH_2+H, 174 (30), 79 (25), 140 (23)
XII	425 (3)	530 (100)	635 (13)	nd	217 (3)	124 (22)	nd	364 (10)	470 (7)	93 (22), 94 (15)
XIII	547 (40)	652 (3)	nd	313 (3)	235 (5)	nd	80 (100)[d]	468 (20)[e]	574 (3)[f]	142 (50), 145 (50), 126 (40), 218 (30), 182 (20), 112 (16)
XIV	456 (100)	561 (37)	666 (3)	241 (10)	216 (3)	156 (40)	nd	396 (7)	502 (5)	126 (60), 183 (17)
XV	555 (87)	660 (23)	765 (10)	241 (21)	nd	156 (66)	160 (18)	396 (57)	502 (23)	126 (100), 183 (44), 141 (27), 368 (24)
XVI	514 (50)	619 (3)	nd	241 (13)	274 (3)	156 (60)	nd	396 (4)	502 (4)	126 (100), 183 (28), 141 (21)
XVII	512 (100)	617 (15)	722 (3)	241 (13)	nd	156 (47)	117 (12)	396 (30)	502 (6)	126 (48), 183 (25), 141 (17)
XVIII	384 (90)	489 (100)	594 (16)	241 (4)	nd	156 (10)	–	–	–	126 (23), 459 (18)
XIX	454 (85)	559 (100)	664 (18)	285 (7)	170 (10)	200 (3)	–	–	–	126 (30), 380 (20), 485 (20)
XX	628 (3)	733 (10)	–	nd	nd	242 (10)	nd	512 (3)	618 (4)	143 (100)
XXI	472 (3)	577 (100)	682 (16)	nd	nd	86 (15)	117 (12)	356 (3)	462 (20)	215 (25), 139 (15), 156 (15), 171 (15)
XXII	576 (3)	681 (100)	786 (7)	nd	nd	190 (30)	117 (32)	460 (8)	566 (20)	215 (47), 156 (45), 112 (43), 85 (37), 139 (37), 171 (37), 99 (27), 218 (23), 537 (20), 432 (15)
XXIII	497 (10)	602 (100)	707 (7)	nd	nd	97 (20)	nd	351 (3)	457 (25)	199 (25), 155 (20), 323 (15)
XXIV	521 (6)	626 (100)	731 (13)	nd	nd	151 (15)	117 (10)	405 (3)	511 (40)	199 (20)

[a] Arranged mainly according to the proposed fragmentation pathways shown in Fig. 2.
[b] Isotopic peaks and peaks of intensities less than 15% were omitted.
[c] Not detectable (intensity less than 3%).
[d] R_2+H.
[e] M–R_2+H.
[f] M+H+DEA–R_2+H.

Table 4
Principal ions[a] in negative FAB mass spectra of 24 cephem antibiotics with DEA

Compound no.	m/z (% I)						
	M–H	M–H+DEA	A_1–H	B	R_2	M–H–CO_2	Others[b]
I	nd[c]	nd	nd	272 (3)	nd	nd	80 (100), 170 (15)
II	524 (4)	nd	196 (10)	nd	172 (100)	nd	
III	453 (8)	nd	166 (15)	184 (25)	131 (100)	409 (2)	69 (50), 58 (15)
IV	461 (3)	nd	190 (10)	208 (10)	115 (100)	nd	
V	nd	nd	nd	nd	115 (100)	nd	
VI	611 (6)	nd	nd	nd	115 (100)	nd	133 (15)
VII	414 (22) 415 (25)[d]	519 (3)	180 (60)	198 (100)	nd	370 (12)	167 (45), 123 (40), 292 (40), 156 (37), 74 (28), 155 (28), 474 (22), 112 (18)
VIII	395 (100)	500 (20)	180 (66)	198 (75)	nd	351 (26)	197 (70), 455 (52), 167 (43), 156 (37), 291 (35), 335 (35), 123 (21), 100 (18)
IX	404 (15)	509 (3)	189 (100)	207 (24)	nd	360 (5)	100 (50), 156 (22), 233 (20), 197 (19)
X	366 (62)	471 (6)	189 (90)	207 (100)	–	322 (80)	153 (62), 233 (57), 176 (54), 286 (52), 159 (45), 426 (38), 100 (27), 82 (20)
XI	346 (61)	451 (3)	189 (95)	207 (80)	–	302 (36)	139 (100), 100 (52), 135 (35), 156 (35), 112 (27), 233 (25), 173 (24)
XII	423 (100)	528 (10)	207 (87)	225 (6)	60 (40)	379 (12)	318 (35), 153 (32), 198 (25), 155 (25), 100 (23), 251 (21), 336 (21), 92 (20):R_1–OCH_3–H, 193 (20), 82 (17)
XIII	545 (5) 546 (6)[d]	nd	311 (5)	nd	nd	nd	103 (100), 124 (82), 82 (38), 100 (27)
XIV	454 (40)	559 (5)	239 (15)	257 (17)	nd	410 (5)	124 (100), 82 (35), 197 (30), 167 (25), 153 (20), 350 (20), 100 (15), 394 (15)
XV	nd	nd	239 (5)	nd	158 (100)	nd	58 (40), 82 (22), 124 (17):R_1–OCH_3–H, 144 (17)
XVI	512 (3)	nd	239 (4)	nd	117 (100)	nd	82 (21), 124 (18)
XVII	510 (5)	nd	239 (4)	nd	115 (100)	nd	
XVIII	382 (45)	487 (5)	239 (8)	257 (20)	–	338 (7)	124 (100), 125 (70):M–H–B, 82 (35), 100 (20)
XIX	452 (30)	557 (8)	283 (10)	301 (3)	–	408 (4)	75 (100), 124 (80), 82 (30), 209 (20), 100 (20)
XX	nd	nd	nd	nd	115 (100)	nd	
XXI	470 (3)	nd	nd	nd	115 (100)	nd	72 (15)
XXII	574 (4)	nd	nd	nd	115 (100)	nd	100 (80), 158 (20), 146 (18)
XXIII	495 (7)	nd	nd	nd	145 (100)	nd	83 (65)
XXIV	nd	nd	nd	nd	115 (100)	nd	

[a] Arranged mainly according to the proposed fragmentation pathways shown in Fig. 2.
[b] Isotopic peaks and peaks of intensities less than 15% were omitted.
[c] Not detectable (intensity less than 2%).
[d] M.

Table 5
Retention time on mass chromatograms of 24 cephem antibiotics using mobile phase containing GLY or DEA

Compound no.	Retention time (min)	
	GLY	DEA
I	12.2	11.4
II	11.7	11.7
III	19.7	15.7
IV	31.0	22.1
V	24.2	17.8
VI	20.9	15.6
VII	15.6	15.6
VIII	34.7	22.7
IX	15.7	15.7
X	14.4	14.6
XI	14.8	14.9
XII	16.9	13.4
XIII	12.0	11.8
XIV	16.1	13.6
XV	16.7	13.8
XVI	32.0	22.3
XVII	18.8	14.1
XVIII	15.6	12.6
XIX	19.2	14.5
XX	20.8	17.4
XXI	21.4	17.2
XXII	16.8	13.9
XXIII	18.3	14.2
XXIV	15.1	11.9

Channels at m/z MH and some major fragment cations were monitored, and time at each peak top was recorded. HPLC–FAB-MS conditions are described in Experimental.

peak of $[M+H]^+$ appeared under the conditions described. Cefazolin sodium was analysed in the positive mode with GLY, the most popular approach in FAB-MS analysis.

The upper panels of Fig. 3 and Fig. 4 show FAB mass spectra of the authentic ceftriaxone sodium and cefazolin sodium, respectively. The lower panel of Fig. 3 shows the mass spectrum obtained from the serum of a subject who had an intravenous injection of 1 g ceftriaxone sodium before sampling, and the lower panel of Fig. 4 shows that of a cadaver who had an intravenous injection of 1 g cefazolin sodium before death. Each spectrum obtained from the two different human sera was almost identical to that of the authentic ceftriaxone sodium or cefazolin sodium, clearly showing the presence of each drug in the serum.

The upper panels of Fig. 5 and Fig. 6 show FAB mass chromatograms for three representative ions of the authentic ceftriaxone sodium and those of the authentic cefazolin sodium, respectively. The lower panels of Fig. 5 and Fig. 6 show mass chromatograms obtained from the same measurements as made in the lower panels of Fig. 3 and Fig. 4, respectively. Big peaks due to impurities appeared on the channels at m/z 133 and 323 approximately 5–11 min of retention time for the extract from the autopsy serum (Fig. 6b). However, no such peaks were observed in the clinical blood (Fig. 5b). Therefore, it seems that they derived from post-mortem changes in the cadaver's blood. Anyway, both ceftriaxone and cefazolin were well separated from impurities (Fig. 5b and Fig. 6b).

From the results described above, capillary HPLC peaks of ceftriaxone and cefazolin corresponding to their peaks on mass chromatograms were composed of pure ceftriaxone and cefazolin, respectively, and were not contaminated by impurities. By UV measurements with the capillary HPLC, a minor modification of the previous study [7], the ceftriaxone or cefazolin concentration in the serum was determined to be 80 $\mu g/ml$ or 70 $\mu g/ml$, respectively.

4. Discussion

In this paper, using a capillary HPLC–FAB-MS system, we have presented both positive and negative ion FAB mass spectra of 24 cephem antibiotics with DEA and GLY as matrices. To our knowledge, no reports have been published on such a systematic analysis of cephem antibiotics with HPLC–FAB-MS. Our on-line HPLC–FAB-MS requires only a simple isolation procedure for all compounds using solid-phase extraction and enables mass spectral analysis of a compound in the extract within the time of its HPLC separation even for the autopsy serum containing much more impurities (Fig. 6). A clean FAB mass spectrum, specific to the compound to be analysed, can be obtained with higher sensitivity by our system, as shown in Fig. 3 and Fig. 4. These are due to not only the general advantages of on-line HPLC–FAB-MS over off-line methods, but also to the advantages of our capillary HPLC–FAB-MS over previous HPLC–FAB-MS utilising the so-called

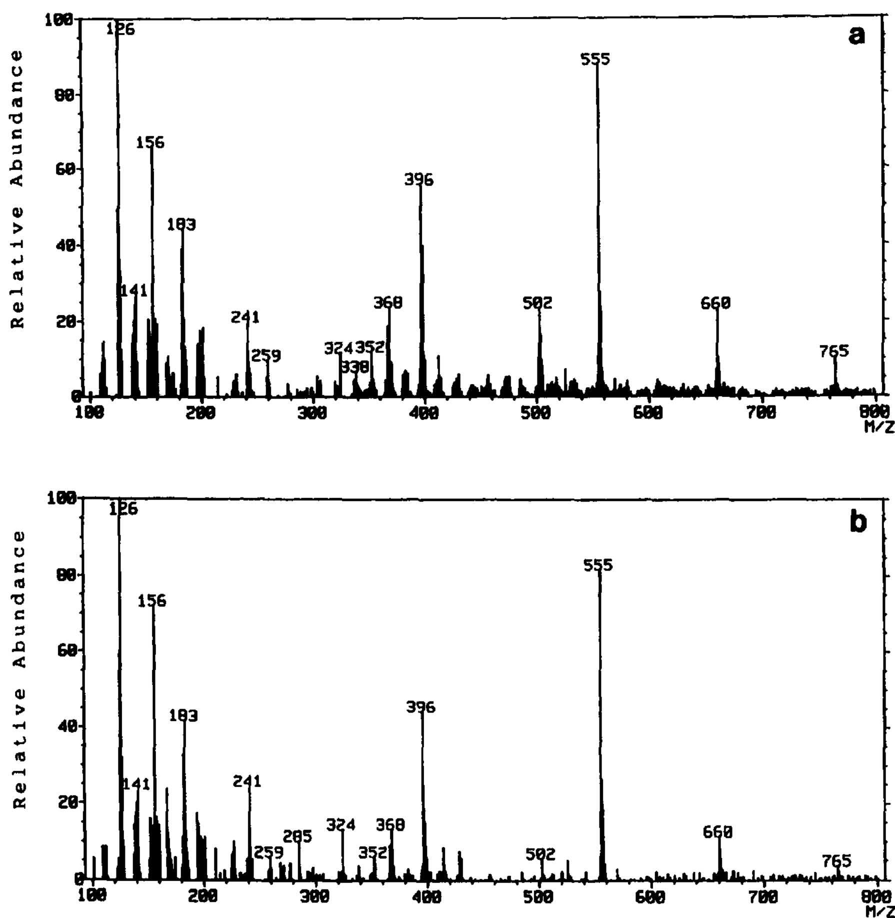

Fig. 3. FAB mass spectra of the authentic ceftriaxone sodium (a) and the extract from serum of a subject who had an intravenous injection of 1 g ceftriaxone sodium 4 h before sampling (b). Measurements were made in the positive mode with DEA. The amount of the authentic ceftriaxone sodium was 5 μg on the column. HPLC–FAB-MS conditions are described in Experimental.

microbore HPLC column (1 mm I.D.) [9,10] as mentioned in detail previously [5,6].

A report on FAB-MS of 14 penicillins indicated that alkali metals strongly affected the patterns of positive FAB mass spectra, but negative spectra were not influenced appreciably by their presence [11]. However, in this report, the presence of alkali metals did not affect the patterns of FAB mass spectra even in the positive mode for either the authentic compounds (Tables 1–4) or the extracts (Fig. 3 and Fig. 4). This seems to be the case mostly because alkali metals are removed from the analytes during the concentration and purification procedure with the column-switching system. On the other hand, neither the quasi-molecular nor molecular anions were detected in the negative FAB mass spectra of several of the cephem antibiotics (Table 2 and Table 4). For some other compounds, the sensitivities of the mass spectral measurements were one order of magnitude lower in the negative than in the positive mode. Therefore, the positive mode should be tried first in the capillary HPLC–FAB-MS analyses of most samples containing cephem antibiotics.

Selection of a matrix among many available materials is important for FAB-MS analysis [12], although GLY is the most widely used. In the positive mode with GLY, intensities of quasi-molecular ions were low for some compounds (Table 1). It has been reported that DEA is useful for molecular mass determination of neutral oligosaccharides con-

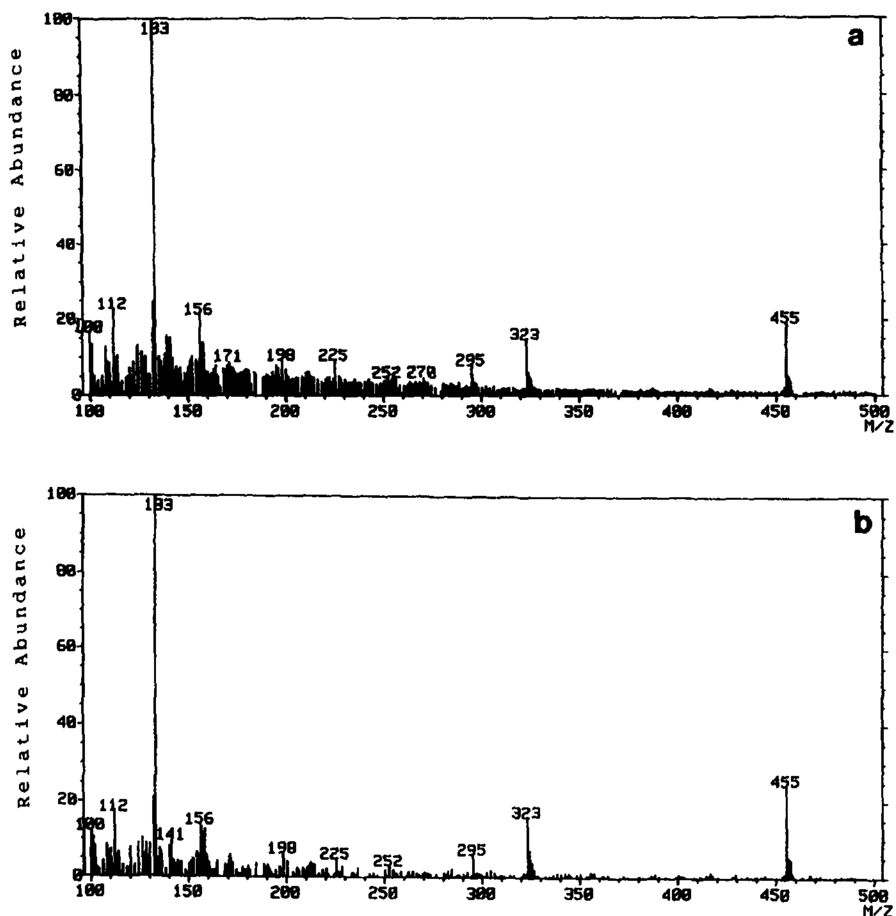

Fig. 4. FAB mass spectra of the authentic cefazolin sodium (a) and the extract from serum of a cadaver who had an intravenous injection of 1 g cefazolin sodium 90 min before death (b). Measurements were made in the positive mode with GLY. The amount of the authentic cefazolin sodium was 2 μg on the column. HPLC–FAB-MS conditions are described in Experimental.

taining macrotetronolide antibiotics because abundant matrix adduct ions at *m/z* M+H+DEA appear in FAB-MS analysis of the compounds [12,13]. Hence, we applied DEA to HPLC–FAB-MS analysis of the cephem antibiotics. This study has confirmed that DEA provides more information on molecular mass of a compound than does GLY, even for acidic compounds such as the cephem antibiotics because of such adduct formation (Table 1 and Table 3).

Many drugs were not clearly separated from each other on mass chromatograms under the present conditions, especially with DEA (Table 5). However, this may not be a significant problem in clinical and forensic analysis because two different cephem antibiotics are not usually administered simultaneously.

Our previous study [5–7] utilized octadecyl columns for capillary HPLC of cephalosporin antibiotics, but the addition of GLY to the mobile phase caused short retention times and low peak heights [7]. The use of phenethyl packing material in the present study improved the retention and peak shape of the cephem antibiotics on the capillary HPLC columns even in the mobile phase containing a matrix, resulting in higher sensitivity. Both the cephem antibiotics and the phenethyl packing materi-

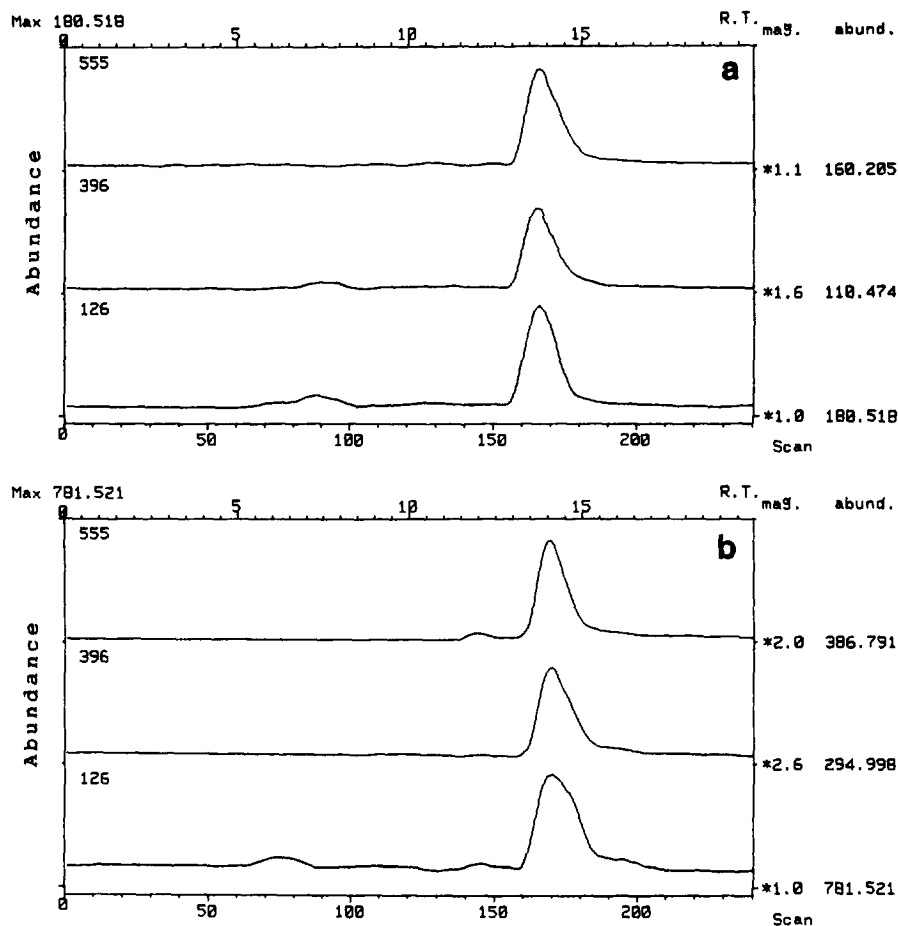

Fig. 5. FAB mass chromatograms for the authentic ceftriaxone sodium (a) and the extract from serum of a subject who had an intravenous injection of 1 g ceftriaxone sodium 4 h before sampling (b). Three representative cations were monitored in the same measurements as made in Fig. 3. R.T., retention time in min.

al have ring structures, and the hydrophobic interactions between them may have been the reason for the improvement.

We frequently encounter cases that require forensic drug screening. The intense peaks due to common moieties shown in Tables 1–4 are a good indication of the presence of a cephem antibiotic. The peaks due to R_1 (m/z 97, 106 and 156), R_1–OCH_3+H (m/z 126), R_2+H (m/z 80) and R_2+2H (m/z 117) in the positive mode, together with the peaks due to R_2 (m/z 115), A_1–H (m/z 180, 189 and 239), B (m/z 198 and 207), M–H–B (m/z 197) and R_1–OCH_3–H (m/z 124) in the negative mode are useful for such indications. Analysing a sample in both positive and negative modes can be very informative for the identification of drugs, and, as such, the data presented in this study will be useful for clinical and forensic laboratories.

The range of the detection limits was rather wide (10 ng–2.5 μg), but no significant differences in the detection limits between analysis with GLY and that with DEA were found for most compounds. It seems certain that the sensitivity is compound-dependent under the present conditions. Nevertheless, the cause of the big differences in the sensitivity among the 24 cephem antibiotics is not presently clear, and future studies are needed to clarify this matter.

Therapeutic serum concentration of cephem antibiotics were reported to be 1–10 μg/ml for oral drugs (IX–XI and XIX), and 40–200 μg/ml for

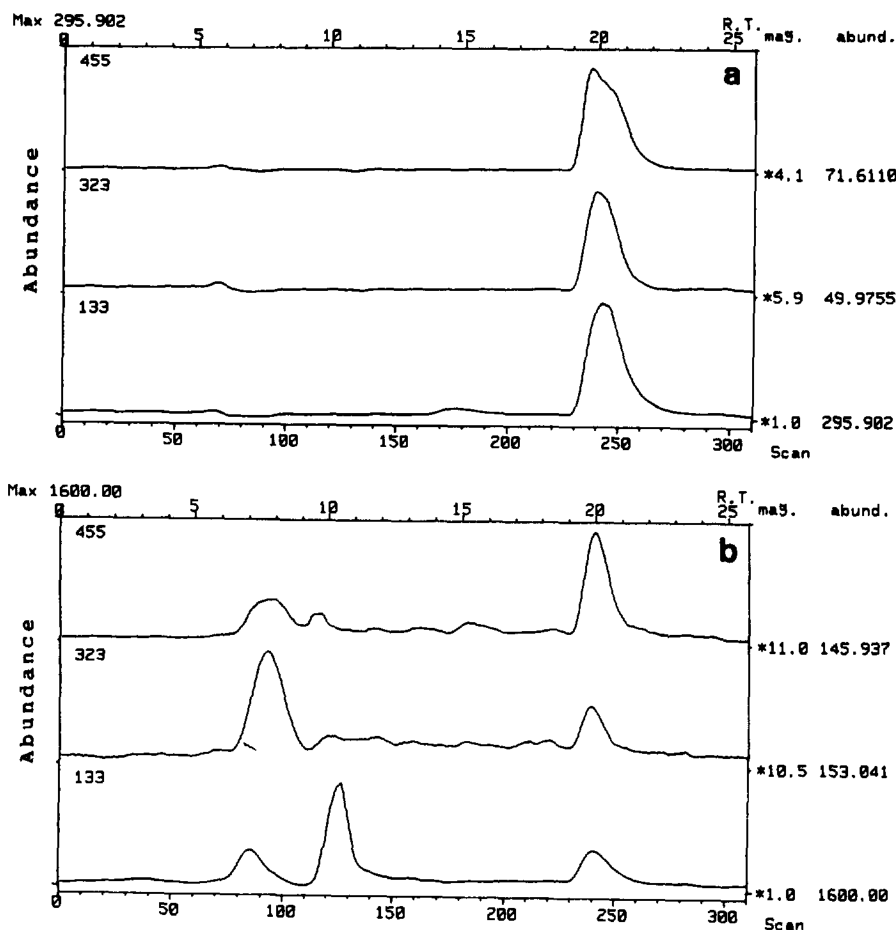

Fig. 6. FAB mass chromatograms for the authentic cefazolin sodium (a) and the extract from serum of a cadaver who had an intravenous injection of 1 g cefazolin sodium before death (b). Three representative cations were monitored in the same measurements as made in Fig. 4. R.T., retention time in min.

injection drugs (others) [14]. We have already reported that two oral drugs (X and XI) showing high sensitivity (10–50 ng on the column) could easily be detected with our previous HPLC–FAB-MS system [5,6]. In the present study, two injection drugs (XV and III) having rather low sensitivities (1–2.5 μg and 200–1000 ng on the column) could also be detected (Fig. 3 and Fig. 4). In addition, recoveries of the 24 cephem antibiotics were generally over 60% for our isolation with the Bond-Elut C_{18} cartridge. Thus, our capillary HPLC–FAB-MS coupled with the isolation procedure seems sufficiently sensitive to detect most cephem antibiotics under investigation.

For all 24 cephem antibiotics, our capillary HPLC–FAB-MS gives clear quasi-molecular peaks together with relatively abundant fragment peaks (Table 1 and Table 3), which are useful for both screening and identification of the drugs [5]. Although the capillary HPLC–FAB-MS is semi-quantitative, accurate quantitation can be achieved if a suitable isotopic internal standard is used.

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