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Jpn. J. Forensic Toxicol. Vol.11, No.3, 1993

原　　報

ANALYSES OF QUINOLONE ANTIMICROBIALS IN HUMAN PLASMA BY CAPILLARY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY／FAST ATOM BOMBARDMENT MASS SPECTROMETRY

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Received July 5, 1993
Accepted October 14, 1993

キャピラリー HPLC/FAB-MS によるヒト血漿中キノロン系抗菌剤の分析

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Summary

Capillary high-performance liquid chromatography (HPLC) was combined with frit fast atom bombardment (FAB)-mass spectrometry (MS), and a detailed procedure has been established for on-line analysis of ten quinolone antimicrobials in human plasma by the HPLC/FAB-MS. A special column switching device for concentration enabled injection of as large as a 500 μl sample; and the capillary column (0.5 mm i.d.) enabled introduction of its entire effluent to the frit interface of FAB-MS. These conditions gave much higher sensitivity than that of the previous HPLC/MS systems. The positive FAB mass spectra of ten quinolone carboxylic acid antimicrobials, measured with the present system, showed peaks at m/z MH, MH+H, MH+32, MH+62, MH+G, MH−H_2O and MH−CO_2 in common for most drugs. The MH^+ quasi-molecular ions constituted base peaks for all drugs. Every drug, which had been spiked to human plasma, could be detected as a clean peak on mass chromatograms with sufficient sensitivity, with use of each base peak. The detection limit of this method was 0.1−0.2 μg/ml plasma for each drug, although the present method is semi-quantitative.

Key words: Quinolone antimicrobials; Pyridonecarboxylic acids; Mass spectrometry; Fast atom bombardment; Liquid chromatography; Capillary HPLC; Frit FAB

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 0915−9606/93/$05.00

Introduction

The on-line combination of high-performance liquid chromatography (HPLC) with mass spectrometry (MS) is now one of the main topics in biological MS studies. With this combination, the final identification by MS has been realized even for polar, involatile and thermolabile compounds, which are not suitable for analysis by gas chromatography (GC)/MS.

The studies on application of HPLC/MS to drug analyses have recently begun, and some reports have been published on cephalexin [1], parathyroid hormone [1], insulin [1], ampicillin [2], penicillin G [3], triazolam [4], chlordiazepoxide and its metabolites [5].

Quinolone carboxylic acid antimicrobials are now one of the most popular drug groups for treatment of bacterial infection. They are usually analyzed by HPLC, because of their high polarity and thermolability [6 −15]. From the medico-legal point of view, their analyses are of interest, because it was reported that concomitant administration of enoxacin and fenbufen induced severe convulsions in several cases [16]. This may be true for other quinolones if they are administered together with fenbufen.

In this paper, we present a detailed procedure for detection of ten quinolone antimicrobials in human plasma by capillary HPLC/frit fast atom bombardment (FAB)-MS. The capillary HPLC column and a special column switching device have given much higher sensitivity for MS identification of drugs.

Experimental

Materials

Chemical structures of ten quinolone antimicrobials tested in the present study are listed in Table 1.

II, IV and V (pure powder each) were obtained from Dainippon Pharmaceutical (Osaka, Japan); I and X from Daiichi Seiyaku (Tokyo, Japan); VIII and IX from Shionogi & Co. (Osaka, Japan); III from Toyama Chemical (Tokyo, Japan); VI from Kyorin Pharmaceutical (Tokyo, Japan); and VII from Bayer AG (Leverkusen, Germany). Other common chemicals used were of the highest purity commercially available. Human plasma was obtained from healthy subjects.

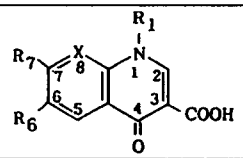
HPLC system

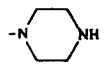
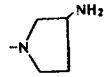
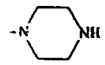
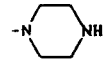
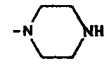
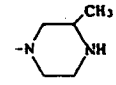
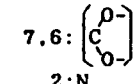
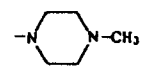
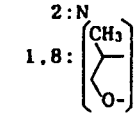
The outline of HPLC and its column switching system is shown in Fig.1. Our system includes two pumps (Shimadzu type LC-9A) for gradient formation and for all other solvent flow until introduction to the MS instrument. To allow a low flow rate through the capillary column, a preinjection split system was adopted with a T- joint and a restriction column (250 x

1.5 mm, 5 μm ODS particle). The capillary HPLC column for separation (250 x 0.5 mm i.d., Develosil Ph-5) contained 5 μm ODS packing material and the concentration column (30 x 0.5 mm i.d., Lop ODS) contained 30 μm ODS material. All columns were purchased from Nomura Chemical Co., Ltd., Seto, Aichi, Japan.

A special column switching loop was devised in our laboratory (see number 6 in Fig. 1). A 100—500 μl sample can be injected into the needle port and sent to the concentration column as shown with the dotted arrows. The drugs thus concentrated on the column were washed with 200 μl of distilled water; after suitable switching of the valves, they are sent to the separation column, followed by introduction to the frit interface, without any loss during the

Table. 1 . Chemical structures of quinolone antimicrobials used in the present study



Compounds (M.W.)	R1	R6	R7	X	Misc.
I.Nalidixic acid (232)	$-C_2H_5$	-H	$-CH_3$	N	-
II.Enoxacin (320)	$-C_2H_5$	-F		N	-
III.Tosufloxacin (404)		-F		N	-
IV.Pipemidic acid (303)	$-C_2H_5$	-		N	6:N
V.Piromidic acid (288)	$-C_2H_5$	-		N	6:N
VI.Norfloxacin (319)	$-C_2H_5$	-F		C	-
VII.Ciprofloxacin (331)		-F		C	-
VIII.Lomefloxacin (351)	$-C_2H_5$	-F		C	8:-F
IX.Cinoxacin (262)	$-C_2H_5$	-	-	C	7,6:  2:N
X.Ofloxacin (361)	-	-F		C	1,8: 

HPLC procedure. All tubings used after the T joint were 0.05 mm in diameter. The flow rate for the capillary separation column was 4 μl/min. A UV variable wavelength detector (Jasco Co, Ltd., Tokyo, Japan) monitored the effluent from the capillary column prior to its introduction to the MS instrument. The mobile phase was methanol/water/acetic acid/glycerin (20−95: 77−2: 2.5: 0.5, v/v, respectively) with a gradient program for change in methanol and water (20 to 50 % methanol in 50 min, followed by 50 to 95 % in 10 min; thereafter 95 %).

FAB MS conditions

The interface between HPLC and the MS was a frit made of a porous stainless filter and fused silica tubing (0.05 mm i.d.). A JMS-DX303 double focusing MS instrument fitted with a FAB ion source (MS-FAB 09A) and a JMA-5000 computer controlled data analysis system (JEOL, Tokyo, Japan) were used. Xenon was used for generation of the fast atom beam with its voltage of 3 keV. The mass spectrometer was scanned over a mass range of m/z 100−800 for 4 s.

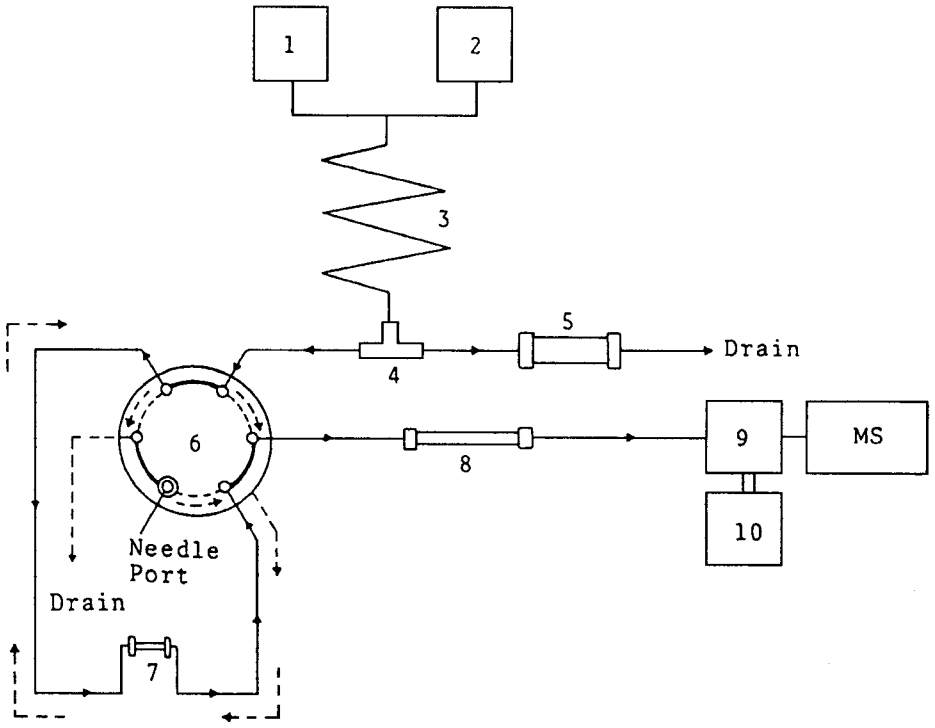


Fig. 1. Schematic diagram of the capillary HPLC system used in the present study. 1 and 2, HPLC pumps; 3, mixing tube; 4, T-joint; 5, restriction column; 6, injector; 7, concentration column; 8, capillary separation column; 9, UV detector; 10, integrator. The dotted arrows show the flow for loading the sample to the concentration column and for its washing.

Spiked samples and their pretreatment

To 1 ml plasma, which had been spiked with quinolone antimicrobials (1 μg each), were added 1 ml distilled water and 0.3 ml perchloric acid. After gentle shaking, it was centrifuged at 800 *g* for 10 min. The supernatant fraction was passed through a membrane filter (pore size: 0.22 mm). The pH of the filtrate was adjusted to 6–8 with 30 % ammonia water or 100 % acetic acid. A 300-μl aliquot of it was injected into the port of the injection loop (Fig. 1).

Results

FAB mass spectra

Table 2 shows positive FAB mass spectra of ten quinolone antimicrobials. They are arranged according to each probable fragmentation or adduct formation pathway. The MH^+ quasi-molecular ions constituted base peaks for all compounds. The $[M+2]^+$ peaks were also relatively

Table. 2. Principal ions in positive FAB mass spectra of ten quinolone antimicrobials

Compound No.	m/z (% I)							
	MH	MH+H	MH+32	MH+62	MH+G	MH-H_2O	MH-CO_2	Others
I	233 (100)	234 (23)	265 (2)	295 (3)	325 (5)	215 (31)	189 (9)	187,379, 465 (9) (8) (4)
II	321 (100)	322 (27)	353 (2)	383 (2)	413 (8)	303 (13)	n.d.*	265,455,234, (6) (3) (3)
III	405 (100)	406 (36)	437 (7)	467 (6)	497 (8)	387 (18)	361 (4)	344,419, 318 (6) (4) (2)
IV	304 (100)	305 (67)	336 (4)	366 (3)	396 (17)	386 (11)	260 (7)	438,227, 276 (5) (4) (2)
V	289 (100)	290 (58)	321 (10)	351 (8)	381 (16)	271 (54)	245 (16)	243,215, 363 (23)(12)(10)
VI	320 (100)	321 (26)	352 (2)	n.d.	412 (8)	302 (11)	276 (12)	264,184,245, 454 (5) (5) (3) (3)
VII	332 (100)	333 (29)	364 (4)	394 (2)	424 (10)	314 (13)	288 (12)	276,466, 231 (5) (5) (3)
VIII	352 (100)	353 (24)	384 (3)	414 (2)	444 (7)	334 (14)	308 (6)	263,282, 486 (5) (3) (2)
IX	263 (100)	264 (28)	295 (2)	325 (1)	355 (6)	245 (8)	219 (6)	409,235,217, 337 (14) (8) (7) (6)
X	362 (100)	363 (32)	394 (12)	n.d.	454 (9)	344 (8)	318 (11)	462, 241 (12) (7)

*n.d., not detectable.

intense for all compounds. Small peaks at m/z MH+32 and MH+62 were observed for most compounds, which are probably due to MH+methanol and MH+ethylene glycol, respectively. The peaks at m/z M−17 and M−43, which probably correspond to MH−H_2O and MH−CO_2, respectively, were also observed for most compounds.

Although we tried to measure negative ion FAB mass spectra for these quinolone antimicrobials, total ion current in the negative mode was too low to be presented with glycerin as matrix.

Detection of drugs by mass chromatography

Figure 2 shows mass chromatograms obtained by HPLC/FAB-MS for the authentic drugs (1 μg each) and a serum sample spiked with the same amount of drugs, with use of each quasi-molecular base peak. With our gradient program, all drugs could be detected within 100 min of retention time.

The recovery of the drugs, which had been added to 1 ml plasma, was 52.3−109 %.

To check quantitativeness of the drugs by the present HPLC/mass chromatography, peak area intensities were plotted against amounts of drugs on column for nalidixic acid, tosufloxacin and lomefloxacin as shown in Fig. 3. The calibration curves were not sufficiently linear, but semi-quantitative. The detection limits of 10 drugs were 0.1−0.2 μg/ml plasma (ca. 10−20 ng on column).

Discussion

To our knowledge, this is the first report dealing with analysis of quinolone antimicrobials in biological samples by combined HPLC/MS.

In this report, we have called the present separation column of 0.5 mm i.d. a "capillary" HPLC column, according to the report of Henzel et al.[17], to distinguish it from so-called microbore or narrow-bore HPLC columns of 1.0−2.1 mm i.d. For the latter columns, post-column splitting is required to provide low flow rates to MS instruments. However, with the present capillary HPLC column, the low flow rates allow introduction of the entire column effluent into the MS instrument without any loss of the sample during analysis. In addition, with a special column switching device for injection and concentration (Fig. 1), up to 0.5 ml samples can be injected for analysis. Both capillary column and large injection capacity have realized a much more sensitive analysis.

Very recently, Sato et al.[4, 5] have reported capillary HPLC/FAB-MS for some benzodiazepines. They used a special micro-pump (Milton Roy, USA) to deliver a 4 μl/min flow to the capillary column. In this study, however, we have used two conventional HPLC pumps

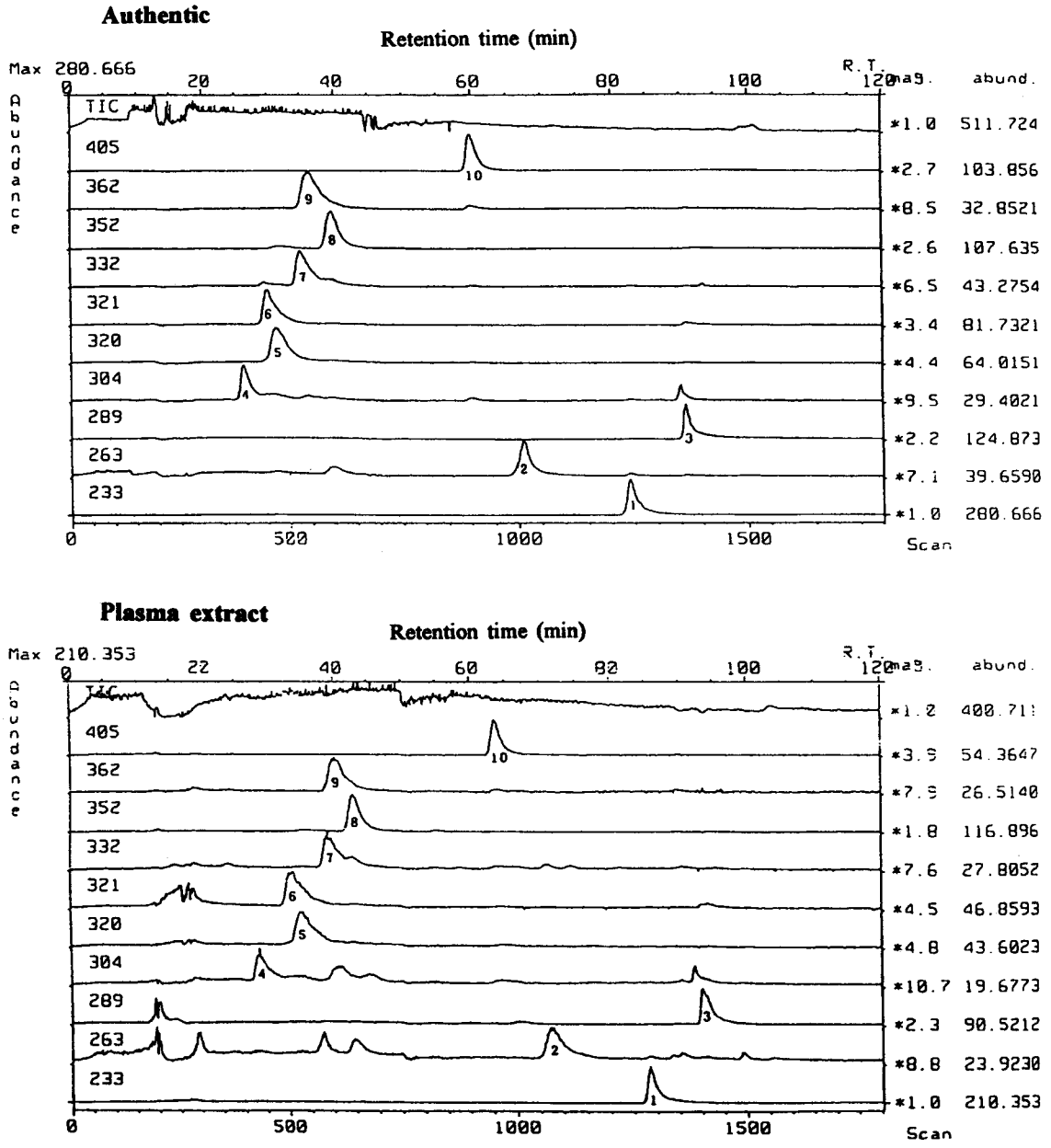


Fig. 2. FAB mass chromatograms for the authentic quinolone antimicrobials (upper) and for spiked plasma (lower). 1, nalidixic acid (I); 2, cinoxacin (IX); 3, piromidic acid (V); 4, pipemidic acid (IV); 5, norfloxacin (VI); 6, enoxacin (II); 7, ciprofloxacin (VII); 8, lomefloxacin (VIII); 9, ofloxacin (II); 10, tosufloxacin (III). The mixture of the ten quinolone antimicrobials, 1 μg of each, was added to 1 ml plasma.

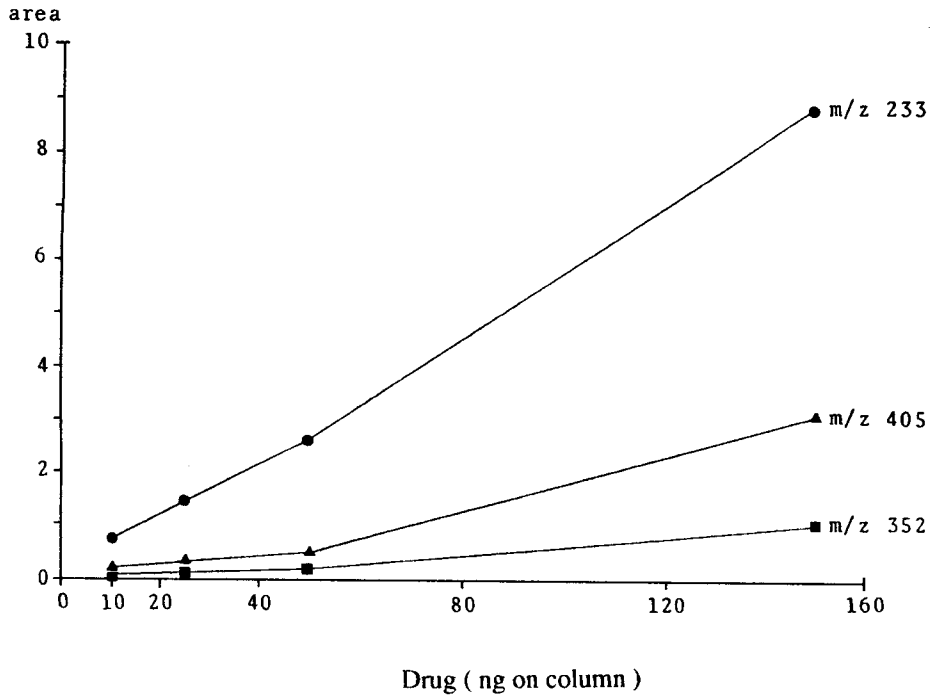


Fig. 3. Calibration curves for nalidixic acid (●), tosufloxacin (▲), and lomefloxacin (■) plotted from peak area intensity and amounts of drugs on column by mass chromatography.

(Shimadzu type LC-9A) designed for semi-microcolumns; to allow a low flow (4 μl/min) through the capillary column, a preinjection split system has been adopted with a T- joint and a restriction column (Fig. 1). This system enables highly reproducible gradient formation and does not require high costs for modification of a conventional HPLC system.

The FAB mass spectra showed base peaks of quasi-molecular ions for all quinolone antimicrobials (Table 2), which seem useful for estimation of molecular weights of drugs. In addition to the quasi-molecular peaks, some fragment peaks appeared; they are very useful for the final identification of a drug.

Therapeutic plasma concentration of quinolone antimicrobials were repoted to be 1−50 μg/ml [18]. The detection limits of the present HPLC/FAB-MS with mass chromatography were 0.1−0.2 μg/ml, although our method is semi-quantitative (Fig. 3). However, with use of suitable isotopic internal standards, accurate quantitation can be made under our conditions. The present method seems very useful in pharmacokinetics, because of its high sensitivity and its ability of the final drug identification. The high sensitivity may be also

useful for trace analyses of drugs in small samples such as blood stains and hair, extending its applicability in forensic and clinical toxicology.

Acknowledgement

This work was partly supported by the Grants in Aid from Ministry of Education, Science and Culture (No. 04670351)

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