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Simultaneous determination of ofloxacin, norfloxacin and ciprofloxacin in human hair by high-performance liquid chromatography and fluorescence detection

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of ofloxacin, norfloxacin and ciprofloxacin in human hair is described. A reversed-phase C_{18} column and a fluorescence detector with switching fluorescence wavelengths were used together with solid-phase extraction of the drugs from hair dissolved in 1 *M* sodium hydroxide. Reproducibility and linearity studies yielded coefficients of variation of 0.2–2.2, 1.4–3.1 and 1.5–3.4%, and correlation coefficients of 1.000, 0.999 and 0.999 within the concentration range 0.3–100 ng/ml for ofloxacin, norfloxacin and ciprofloxacin, respectively. For validation, hair samples were obtained from six subjects who had been taking one or two of the three fluoroquinolones. Assuming a hair growth-rate of 1 cm per month fluoroquinolones could be detected in the hair section(s) that had grown approximately between the dates of drug administration and hair sampling.

1. Introduction

According to several studies [1–4], hair analysis offers the possibility to reveal an individual's history of exposure to drugs or heavy metals, going back from the sampling time and dating back over a period of months or years. In our previous reports [5–10] we suggested the potential usefulness of analyzing the axial distribution of a drug along a single hair shaft, because it allows investigation of the individual's history of drug use, and even patient compliance with drug therapy, which is one of the most important pieces of information in rational drug therapy. Furthermore, we found that ofloxacin, one of the most widely used antimicrobial fluoroquinolone derivatives in the world, could be detected in a single hair even after only a short exposure over one to several days [11–13]. Thus, we propose to utilize ofloxacin as a "time marker" along the hair shaft for analyzing other drug(s) in hair. In fact, the uncertainty on each single hair's growth rate and growth stage, both of which are quite variable within and between subjects [13,14], may negate the information obtained from the analysis of a single hair [15].

Many new fluoroquinolone derivatives have been developed or are under development in Japan because of their potent and wide-spectrum antimicrobial activities, and good clinical efficacy [16]. Thus, at present we have many drugs that may serve as time markers in hair. Among those

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fluoroquinolones, in particular ofloxacin, norfloxacin and ciprofloxacin are frequently prescribed in Japan for the treatment of various types of microbial infections.

In the present study, we have developed a method for the simultaneous determination of ofloxacin, norfloxacin and ciprofloxacin in a single hair sample, which can be used to assess whether or not these drugs can be detected in a single hair after exposure over a few to several days, and whether the determination of these drugs along a single hair can serve as time markers in hair.

2. Experimental

2.1. Chemicals

All organic solvents and other chemicals were of HPLC grade. Ofloxacin and (*R*)-9-fluoro-2,3-dihydro-3-methyl-10-(4-ethyl-1-piperazinyl)-7-oxo-7H-pyridol[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid (DS-4632), which was used as an internal standard (I.S.), were kindly donated by Daiichi Pharmaceutical (Tokyo, Japan). Norfloxacin and ciprofloxacin were also kind gifts from Kyorin Pharmaceutical (Tokyo, Japan) and Bayer Yakuhin (Osaka, Japan), respectively.

2.2. Apparatus and chromatographic conditions

The HPLC method described here was developed using a Shimadzu (Tokyo, Japan) HPLC system, consisting of a liquid chromatograph (LC9A), an automatic injector (SIL-6B), a system controller (SCL-6B), a column oven (CTO-6A), a fluorescence spectrophotometer (Model RF-5000) and an integrator (CR-4A). A reversed-phase column (TSKgel ODS-80Ts, Tosoh, Tokyo, Japan; 150 × 4.6 mm I.D.; 5 μm particle size) was used as the analytical column. Ofloxacin, I.S., norfloxacin and ciprofloxacin were eluted isocratically using a mobile phase consisting of acetonitrile–0.025 *M* orthophosphoric acid (adjusted to pH 3.0 with 0.5 *M* tetra-*n*-butylammonium hydroxide solution) (5:95, v/v). The solution was filtered and degassed before use. The HPLC system was operated at 40°C and the flow-rate was 1 ml/min.

The excitation and emission wavelengths were as follows: 295 nm and 490 nm, respectively, for ofloxacin, and 280 nm and 445 nm, respectively for I.S., norfloxacin and ciprofloxacin. The wavelengths were first adjusted for the determination of ofloxacin and then automatically switched to those used for the determination of I.S., norfloxacin and ciprofloxacin in a series of analyses. Quantification for each of the three drugs was performed by plotting the peak-area ratio (analyte/I.S.) against the drug concentration to construct a standard curve and by comparing the results of each sample with each obtained curve.

For constructing each calibration curve, blank hair collected from subjects who had not taken any of the three fluoroquinolones for at least several months prior to hair sampling was spiked with a drug and the same extraction procedure as described below was employed.

2.3. Preparation of standard solutions

Stock solutions (1.0 mg/ml) of the three quinolones and the internal standard were prepared in 0.25 *M* orthophosphoric acid. Standard solutions containing these quinolones in the concentration range 3.1–1000 ng/ml were prepared by diluting the stock solution with water. These were then added to blank hair dissolved in 1 *M* NaOH to give the required standard concentrations. The internal standard solution was diluted with water to a final concentration of 1 μg/ml.

2.4. Analytical procedures

Hair samples were washed and treated in the same way as described previously [11]. In brief, hairs were washed successively with a 0.1% solution of sodium dodecyl sulfate and distilled water for 30 min and this procedure was repeated twice. The washed hairs were blotted between two sheets of paper towel and allowed

to dry at room temperature. A single hair was sectioned serially into 1-cm long fragments starting from the hair bulb. Each hair fragment was dissolved in 0.5 ml of 1 *M* NaOH by heating at 80°C for 30 min. After cooling, the solution was neutralized with 0.5 ml of 1 *M* HCl and 1 ml of 0.1 *M* potassium hydrogen citrate buffer (pH 4.6) and 0.05 ml of I.S. solution in water (1 μg/ml) were successively added. Solid-phase extraction of the fluoroquinolones from the above hair solution was carried out by passing the mixture through a C_8 cartridge (Bond Elut C_8 column, Varian, Harbor City, CA, USA) and by eluting with 2 ml of tetrahydrofuran–0.25 *M* orthophosphoric acid (2:8, v/v), according to the procedures described by Okazaki *et al.* [17]. The eluate was evaporated to dryness with a concentrator (SpeedVac Concentrator, Savant Instruments, Farmingdal, NY, USA). The residue was dissolved in 150 μl of the mobile phase using a vortex-mixer. A 60-μl aliquot was then injected onto the column.

2.5. Application in real cases

The applicability of the method was demonstrated in six patients (age: 20–50 years), who had taken one or two of the fluoroquinolones at doses of 300–600 mg/day for 3 to 23 consecutive days. The 1-cm long hair section, in which these quinolones peaked, was determined. Then, a 3–4-cm long section of hair, with the above 1-cm section at its middle, was cut from another hair shaft and sectioned into 2-mm-long fragments serially from the scalp side. Again, the quinolones in each fragment of hair were measured. Since it has been shown that the axial distribution of ofloxacin along a shed hair was quite different from that along a plucked hair collected from the same subject, because the shed hair has been in a resting stage for a few months [18], only plucked hairs with a visible bulb were used for the present analysis. In practice, several strands of hair were sampled by plucking from the occipital area of each patient after having obtained his informed consent.

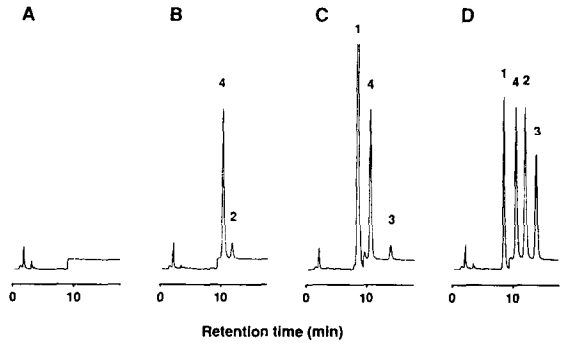

Fig. 1. Chromatograms of blank hair (A), hairs sampled from patients who had been taking 300 mg/day norfloxacin for 10 days (B), hairs sampled from patients who had been taking both 300 mg/day ofloxacin for 10 days and 300 mg/day ciprofloxacin for 5 days at an interval of one week (C), and blank hair supplemented with ofloxacin (5 ng; peak 1), norfloxacin (5 ng; peak 2), ciprofloxacin (5 ng; peak 3) and an internal standard (DS-4632, 50 ng; peak 4) (D). The wavelengths were first adjusted for determination of ofloxacin and then automatically switched to those used for determination of I.S., norfloxacin and ciprofloxacin.

3. Results

3.1. Chromatography

Typical chromatograms are shown in Fig. 1. The chromatogram of blank hair was free from interferences by endogenous compounds, especially around the retention times of the three fluoroquinolones and the I.S. (Fig. 1A). Under the described HPLC conditions, a chromatographic run was performed within 15 min. Ofloxacin, I.S., norfloxacin and ciprofloxacin appeared in this order at retention times of 8.1, 10.2, 11.7 and 13.5 min, respectively, and the separation of the peaks from one another was sufficient (Fig. 1D). Representative chromatograms of real hair samples are shown in Fig. 1B and C.

3.2. Linearity

Standard curves were constructed by fitting the peak-area ratio of a drug to that of I.S. against the spiked blanks with known concentrations of the drugs, according to a linear least-square

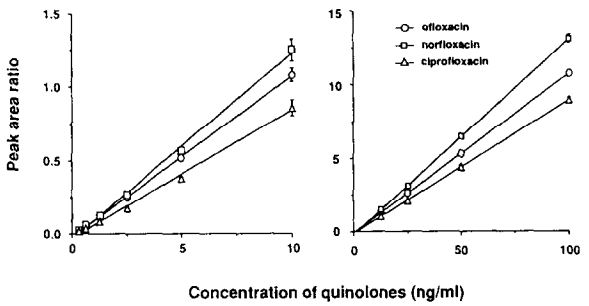

Fig. 2. Calibration curves for three fluoroquinolones in human hair determined by HPLC. All data were expressed as means ± S.D. ($n = 5$).

regression (Fig. 2). For all three fluoroquinolones, linear curves were obtained within the concentration range 0.3–100 ng/ml, and the mathematical expressions and correlation coefficients were as follows: ofloxacin, $y = 0.0185 + 0.107x$ ($r = 1.000$); norfloxacin, $y = -0.0690 + 0.131x$ ($r = 0.999$); ciprofloxacin, $y = -0.0529 + 0.894x$ ($r = 0.999$). Detection limits were determined to be 0.2 ng/ml for ofloxacin and norfloxacin, with coefficients of variation of 10.0% and 9.9%, respectively, and 0.3 ng/ml for ciprofloxacin, with a coefficient of variation of 9.4%.

3.3. Assay accuracy and precision, and extraction recovery

The accuracy, precision and extraction recovery of the measurement of each fluoroquinolone were determined as follows: each drug was added to the blank hair dissolved in 1 *M* NaOH (0.3 mg/0.5 ml) in final concentrations of 12.5, 50 and 100 ng/ml. These spiked samples ($n = 4$ for each concentration) were treated in the same way as described above, including the solid-phase extraction procedure. Accuracy was calculated as the percentage difference between the amount of drug added to the blank hair and the amount of drug measured. The coefficients of variation thus obtained are shown in Table 1.

The extraction recovery was determined by comparing the peak area of an extracted spiked sample with that of a direct injection of an aqueous solution containing the same concentration of drug (Table 1). The over-all recoveries within the concentration range determined were 78.4–95.4% for ofloxacin, 80.7–92.1% for norfloxacin, and 77.6–88.9% for ciprofloxacin, respectively.

The intra-day precision was assessed by carrying four replicate samples of three concentra-

Table 1
Accuracy, precision and recovery of quinolones

Compound	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy[a] (%)	Precision[b] (%)	Recovery[a] (%)
Ofloxacin	12.5	12.6 ± 0.02	101.4 ± 0.19	0.2	87.2 ± 5.02
	50	49.7 ± 0.24	99.4 ± 0.48	0.5	83.1 ± 2.71
	100	100.1 ± 2.18	100.1 ± 2.53	2.2	83.5 ± 3.23
Norfloxacin	12.5	12.8 ± 0.18	100.8 ± 1.39	1.4	84.7 ± 4.59
	50	49.9 ± 1.56	99.7 ± 3.15	3.1	85.4 ± 0.31
	100	100.1 ± 2.04	100.1 ± 2.05	2.0	86.4 ± 3.29
Ciprofloxacin	12.5	12.8 ± 0.20	101.0 ± 1.60	1.5	81.7 ± 4.43
	50	49.9 ± 1.67	99.5 ± 3.36	3.4	84.1 ± 0.28
	100	100.1 ± 1.94	100.1 ± 1.95	1.9	85.2 ± 3.16

[a] All data are expressed as mean ± S.D. ($n = 4$).
[b] Coefficient of variation.

tions through the entire procedure in a one-day analysis. The results on the reproducibility are summarized in Table 1. The coefficients of variation ranged from 0.2 to 3.4% in the case of the spiked standard.

3.4. Application

The cm-by-cm analysis of the fluoroquinolone content along a single hair shaft was carried out in hair samples collected from 6 patients, as previously described. Thus, the distance from the scalp of the hair sections retaining the quinolone was roughly determined in each subject (Fig. 3, upper panels). In all the subjects, the axial distribution of the drug along a single hair shaft coincided approximately with the history of drug use, assuming a hair growth-rate of *ca.* 1.0 cm per month. Then, a 3- or 4-cm long section obtained from another hair strand, with the above determined distance at its middle, was sectioned into 2-mm long fragments to determine the quinolone concentrations in each fragment (Fig. 3, lower panels). This analysis showed that the drug distributed along only four to five consecutive 2-mm long fragments and the hair fragment with the highest drug content closely coincided with the date of drug use.

4. Discussion

The method described here allowed a simple, reproducible and simultaneous determination of three widely-used fluoroquinolones, *i.e.* ofloxacin, norfloxacin and ciprofloxacin, in human hair samples without any interferences from endogenous hair components.

To establish a highly sensitive method enabling us to quantitatively analyze the distribution of these fluoroquinolines along only a single hair shaft, we modified our previous method [11] by using a counter-ion (tetra-*n*-butylammonium hydroxide solution) in the chromatographic sepa-

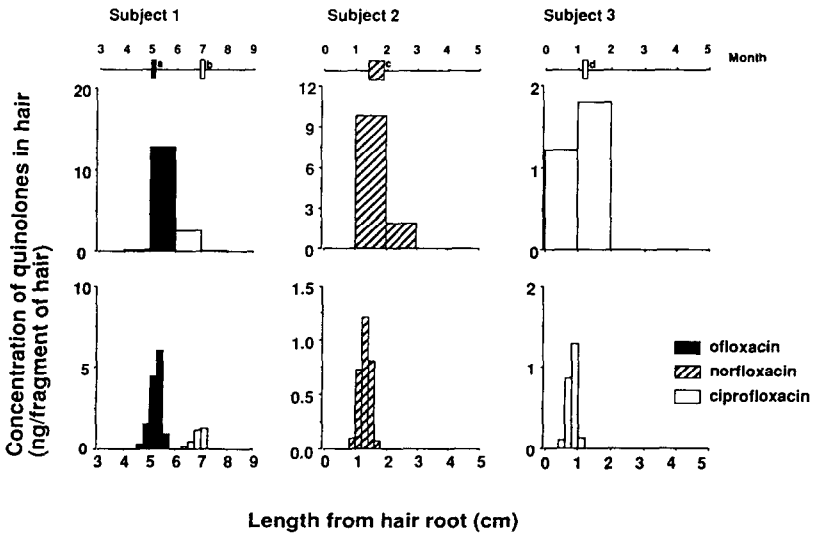

Fig. 3. Axial distribution of fluoroquinolones along a single hair. After the cm-by-cm axial distribution of quinolones was determined by using a single hair (upper panels), the detailed distribution was determined separately in another strand of hair (lower panels). The results of three representative patients' hairs are described. *The drug content in each hair fragment is expressed as the height of column and is shown for 3 representative subjects.* The axes at the top of the figures represent the month before hair collection. The dosage history is depicted above each panel by bars and letters (a–d), assuming a hair growth rate of 1 cm per month as follows: subject 1, a: ofloxacin, 300 mg per day for three days; b: ciprofloxacin, 300 mg per day for three days; subject 2, c: norfloxacin, 600 mg per day for fourteen days; subject 3, d: ciprofloxacin, 400 mg per day for three days.

ration, solid-phase extraction, and on-line switching of wavelengths in the fluorimetric detection. As compared with our previous method, in which liquid–liquid extraction was utilized and the peaks of norfloxacin and ciprofloxacin could not be sufficiently separated from interfering peaks due to unknown hair components, the present method allows a better recovery, *i.e.* 82–87% (Table 1) *vs.* 53–58% in the previous method, and gives remarkably clean chromatograms of blank hair (Fig. 1A). Switching of the detection wavelengths according to the specific fluorescence spectra of each analyte (and I.S.) greatly improved the sensitivity, and thus the three quinolones could be simultaneously detected from only a 2-mm long fragment of hair.

The applicability of the present method was tested using hair samples collected from patients who had used one or two of these three fluoroquinolones a few months before hair sampling. Each drug could be detected in those 1-cm long section(s) of a single hair, that correspond approximately with the hair growth between the dates of drug intake and hair sampling, assuming a hair growth-rate of 1 cm/month. These results suggest that norfloxacin and ciprofloxacin, as well as ofloxacin, could serve as time-markers along a single hair shaft. We have so far shown that the dosage history of antipsychotic haloperidol or chlorpromazine can be deduced by analyzing the axial drug distribution along a single hair shaft [8,19]. However, the uncertainty in the growth rate and the stage of the analyzed hair may negate the obtained result since hair growth is not synchronous, *i.e.* the growth rate is variable both within and between individuals, ranging from 0.5 to 2.0 cm/month, and the resting stage may continue for as long as 3 months [14,18]. The detailed analysis of a single hair revealed that the drug distributed along 4 to 5 consecutive 2-mm long fragments having an obvious peak in drug content, which closely coincided with the date of drug intake (Fig. 3 lower panels). The result that the detailed distribution of fluoroquinolone along a single hair shaft acts as a time marker seems to strongly promote our idea to reveal the history of drug use by analyzing only a single hair. Moreover, the smaller the number of hair strands used, the less invasive the examination for the patient.

Since these drugs are widely used all over the world this kind of analysis of fluoroquinolone may be useful not only in clinical pharmacology but also in the field of forensic science and toxicology. For example, it may be possible to deduce the approximate date of a murder from analysis of the drug distribution along a hair shaft sampled from the victim, even when the remains are found to be nothing more than bleached bone possessing only hair after abandonment of the dead body for a long period of time, when the victim is known to have been taking one of these fluoroquinolones.

5. Conclusion

We describe here a simple, sensitive, and accurate HPLC method for the simultaneous measurement of ofloxacin, norfloxacin and ciprofloxacin in human hair samples. The retrospective analysis of the fluoroquinolone distribution along a single hair shaft revealed the exact individual history of exposure to the drug and, furthermore, that the hair section containing the fluoroquinolone may act as a time marker along that hair shaft.

6. References

[1] A.M. Baumgartner, P.F. Jones and C.T. Black, *J. Forensic Sci.*, 26 (1981) 576.
[2] O. Suzuki, H. Hattori and M. Asano, *J. Forensic Sci.*, 29 (1984) 611.
[3] D. Airey, *Sci. Total Environ.*, 31 (1983) 157.
[4] S. Balbanova, H. Brunner and R. Nowak, *R.Z. Rechtmed.*, 98 (1987) 229.
[5] T. Uematsu, R. Sato, K. Suzuki, S. Yamaguchi and M. Nakashima, *Eur. J. Clin. Pharmacol.*, 37 (1989) 239.
[6] R. Sato, T. Uematsu, R. Sato, S. Yamaguchi and M. Nakashima, *Ther. Drug Monit.*, 11 (1989) 686.
[7] T. Uematsu and R. Sato, *Ther. Drug Monit.*, 12 (1990) 582.
[8] H. Matsuno, T. Uematsu and M. Nakashima, *Br. J. Clin. Pharmacol.*, 29 (1990) 187.
[9] T. Uematsu, R. Sato, O. Fujimori and M. Nakashima, *Arch. Dermatol. Res.*, 282 (1990) 120.

[10] A. Mizuno, T. Uematsu, A. Oshima, M. Nakamura and M. Nakashima, *Ther. Drug Monit.*, 15 (1993) 99.
[11] N. Miyazawa, T. Uematsu, A. Mizuno, S. Nagashima and M. Nakashima, *Forensic Sci. Int.*, 51 (1991) 65.
[12] T. Uematsu, N. Miyazawa and M. Nakashima, *Eur. J. Clin. Pharmacol.*, 40 (1991) 581.
[13] N. Miyazawa and T. Uematsu, *Ther. Drug Monit.*, 14 (1992) 525.
[14] M. Saitoh, M. Uzuka, M. Sakamoto and T. Kobori, in W. Montagna and R.L. Dobson (Editors), *Advances in Biology of Skin, Vol. 5*, Pergamon, New York, 1967.
[15] T. Uematsu, *Clin. Pharmacokin.*, 25 (1993) 83.
[16] T. Uematsu and M. Nakashima, in S. Mitsuhashi (Editor), *Progress in Drug Research*, Vol. 38, Birkhaeuser Verlag, Basel, 1992, pp. 39.
[17] O. Okazaki, H. Aoki and H. Hakusui, *J. Chromatogr.*, 563 (1991) 313.
[18] T. Uematsu, *Forensic Sci. Int.*, in press.
[19] H. Sato, T. Uematsu, K. Yamada and M. Nakashima, *Eur. J. Clin. Pharmacol.*, 44 (1993) 439.