*Journal of Chromatography*, 564 (1991) 171–179
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5665

# Automated analysis of oxolinic acid and flumequine in salmon whole blood and plasma using dialysis combined with trace enrichment as on-line sample preparation for high-performance liquid chromatography

TONE AGASØSTER and KNUT E. RASMUSSEN*

*Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo 3 (Norway)*

(First received August 21st, 1990; revised manuscript received September 28th, 1990)

## ABSTRACT

The use of dialysis as sample clean-up for high-performance liquid chromatography makes fully automated determination of drugs in whole blood and plasma possible. High recoveries of the analytes oxolinic acid and flumequine and the internal standard nalidixic acid are obtained after a short time of dialysis (7.3 min). The dilute dialysates are enriched on a small column packed with polystyrene. When dialysis is discontinued, the analytes are eluted by mobile phase to the analytical column. With UV detection the limit of detection was 50 ng/ml for both oxolinic acid and flumequine. Validation showed good precision and accuracy and good correlation between determinations in plasma and whole blood.

## INTRODUCTION

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid) (OA) and flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1*H*,5*H*-benzo[*ij*]quinolizine-2-carboxylic acid (FQ) are synthetic antibacterials active against gram-negative bacteria. Traditionally, drugs from this chemical class, the quinolone derivatives, have been applied against urinary tract infections in humans. In recent years OA and FQ have also been introduced in the treatment of bacterial infections in salmon and rainbow trout, especially in the case of the feared disease furunculosis in farmed fish.

The pharmacokinetics of these drugs in fish has not yet been fully elucidated. Pharmacokinetic studies have to be carried out to evaluate drug dosage forms and to determine the optimal dosage regimens. Analytical methods for pharmacokinetic studies should be selective, rapid and easy to carry out, because samples may contain metabolites of the drug and the number of samples is large. Regarding selectivity, high-performance liquid chromatographic (HPLC) methods are superior to microbiological and fluorimetric techniques.

0378-4347/91/$03.50 

Several HPLC methods for the determination of OA and FQ in plasma and serum have been published. Manual methods include sample preparation by liquid–liquid extraction [1–3] or solid-phase extraction and subsequent evaporation and reconstitution [4]. All these methods are cumbersome and time-consuming. Two automated methods have been published [5]. They are based on solid-phase extraction and column switching, respectively, and are well suited for determination in plasma.

In field studies whole blood from fish is often frozen, stored, and sent to the laboratory for analysis. The samples hemolyse when they are frozen and neither plasma nor serum can be obtained. This, and the fact that blood volumes available in fish are limited, makes the determination of the drugs in whole blood of interest. No method for the determination of OA or FQ in whole blood appears to be available.

This paper describes a rapid and simple HPLC method for analysis of OA and FQ in plasma and whole blood. The sample preparation is carried out by the combination of dialysis and trace enrichment using the ASTED™ system (Automated Sequential Trace Enrichment of Dialysates) connected on-line with the HPLC system.

## EXPERIMENTAL

### *Chemicals and reagents*

OA and the internal standard, nalidixic acid (NA) were obtained from Sigma (St. Louis, MO, U.S.A.) and FQ from Solchem Italiana (Mulazzano, Italy). Sodium dihydrogenphosphate, orthophosphoric acid and Triton® X-100 were obtained from Merck (Darmstadt, Germany) and sodium hydroxide from EKA (Bohus, Sweden). HPLC-grade acetonitrile and tetrahydrofuran were from May & Baker (Dagenham, U.K.) and Rathburn (Walkerburn, U.K.), respectively. Water was purified in a Milli-Q system (Millipore, Milford, MA, U.S.A.).

### *Preparation of standards*

Standard solutions of OA and FQ of 1 mg/ml were prepared in 0.01 *M* sodium hydroxide. These solutions were kept at 4°C. Citrated blood was used. Plasma was prepared by centrifugating blood twice for 15 min at 1920 *g*. Whole blood and plasma were stored at −20°C. Spiked standards were prepared from thawed blood or plasma and the standard solutions.

### *Internal standard solution*

Nalidixic acid from a solution of 1 mg/ml in 0.01 *M* sodium hydroxide was diluted with 1 *M* trichloroacetic acid and 1 *M* phosphate buffer (pH 7.0) in the ratio of 1:4 to a concentration of 5.0 μg/ml.

*Chromatography*

The chromatographic system consisted of an isocratic pump, LC-6A (Shimadzu, Kyoto, Japan), a UV–visible spectrophotometric detector, SPD-6A (Shimadzu) set at a wavelength of 325 nm, a polystyrene analytical column PLRP-S (particle size 5 μm, 150 mm x 4.6 mm I.D.) (Polymer Labs. Church Stretton, U.K.) and a Chromatopac C-R3A integrator (Shimadzu) where peak heights were recorded. The mobile phase, acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (20:14:66, v/v) was pumped at a flow-rate of 0.7 ml/min.

*Dialysis and trace enrichment*

The ASTED system (Gilson Medical Electronics, Villiers-le-Bel, France) comprised a sample injector (Model 231), two dilutors (low-pressure pumps) (Model 401) equipped with 1-ml syringes, all controlled from a keypad that is part of the Model 231, plus a dialysis cell with a donor volume of 100 μl fitted with a dialysis membrane made of cellulose (Cuprophan™) with a molecular mass cut-off of 15 000. A column (10 mm x 2.0 mm I.D.) (Chrompack, Middelburg, The Netherlands) packed with 36 μm polystyrene (Dynospheres, Dyno Particles, Lillestrøm, Norway) was mounted on a six-port valve (Rheodyne, Berkeley, CA, U.S.A.) Model 7010 that connected the trace enrichment column to the recipient channel of the dialysis cell or to the HPLC system when the valve was switched.

*ASTED sample preparation procedure*

Schematic diagrams of the ASTED system are shown in refs. 6 and 7: the present set-up differed only in that the HPLC mobile phase was back-flushed through the trace enrichment column. Through the first dilutor 100 μl of sample were mixed with 100 μl of internal standard solution, and the donor channel of 100 μl over-filled with this mixture. There it was kept static for 7.3 min while 4 ml of 0.02 *M* phosphate buffer pH 5 were pumped through the recipient channel in pulses by the second dilutor, which simultaneously pumped the dialysate into the trace enrichment column. The pulsation was accomplished by pumping 23 portions of 175 μl (the volume of the recipient channel) into the dialyser, the second dilutor being programmed to pump 1.7 ml/min, and keeping them static for *ca.* 13 s. Subsequently the analytes and the internal standard were eluted from the trace enrichment column to the analytical column by back-flushing with HPLC mobile phase for 2 min. At the same time, residual sample and dialysate in the dialyser were purged out by the two dilutors using 2 ml of the respective solutions, the priming solution for the donor side containing a surfactant, Triton X-100, at a concentration of 0.01% (w/v). Following elution the trace enrichment column was regenerated with 2 ml of recipient solution, and the ASTED system was ready to prepare the second sample while the first sample was being analysed. The second sample was ready for injection just before the analysis of the first sample was finished, the analysis time being 13.5 min.

## RESULTS AND DISCUSSION

### *HPLC separation*

The HPLC conditions used are discussed elsewhere [5]. Similar conditions have also been applied for muscle extracts of OA and FQ [8], and also in part in refs. 9 and 10. In the present method a minor modification in the composition of the mobile phase was made: the concentration of orthophosphoric acid was increased from 65 to 66% (v/v) and the concentration of tetrahydrofuran lowered accordingly to improve the separation of NA and FQ.

Chromatograms obtained after dialysis of salmon whole blood and plasma spiked with 1 μg/ml of OA and FQ and drug-free whole blood and plasma are showed in Fig. 1. The blanks did not contain substances that would interfere in the detection of OA and FQ or the internal standard at 325 nm, but if the detector was set at the absorption maximum of the drugs, 260 nm, interference was observed. The wavelength of 325 nm and other values in this region have been used as excitation wavelengths in fluorescence detection of OA and FQ [3,8,11].

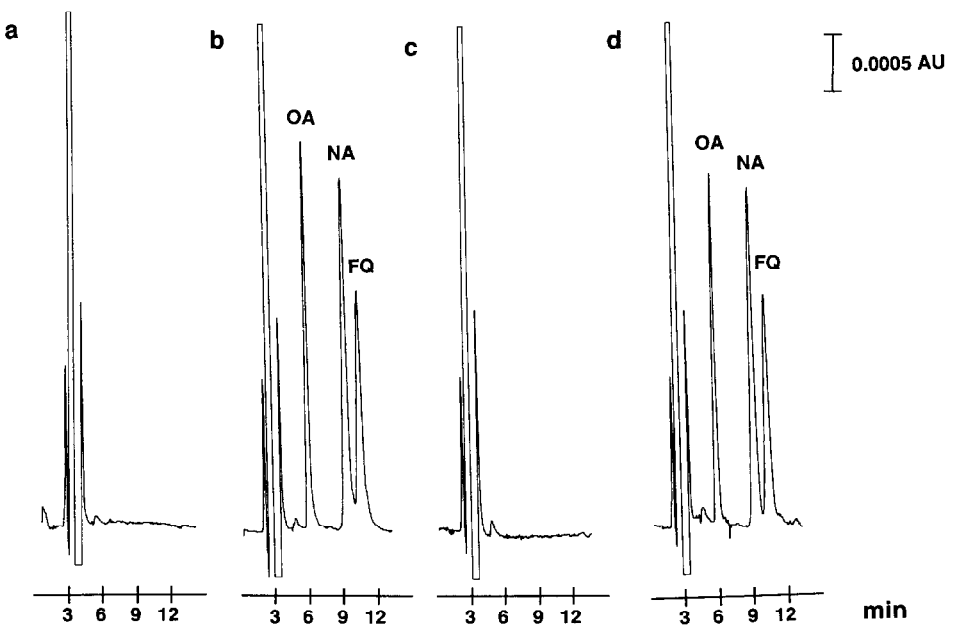


Fig. 1. Chromatograms after addition of internal standard reagent and dialysis of (a) drug-free salmon plasma (internal standard reagent without NA), (b) salmon plasma spiked with 1 μg/ml OA and FQ (internal standard reagent containing 1 μg/ml NA), (c) drug-free salmon whole blood (internal standard reagent without NA) and (d) salmon whole blood spiked with 1 μg/ml OA and FQ (internal standard reagent containing 1 μg/ml NA). Detection wavelength, 325 nm. For other conditions see text.

*Factors governing dialysis efficiency*

Dialysis is a process in which molecules are separated by a semipermeable membrane on the basis of differences in molecular size. The technique is well suited for separating unbound drug molecules from macromolecular proteins and particles in biological samples, using membranes with molecular mass cut-offs of 10 000 or 15 000. Fick's laws describe how the flow of a solute that is able to traverse the membrane is related to the diffusivity of the solute, to the membrane area available for diffusion and to the concentration gradient across the membrane [12]. In the ASTED dialysis cell the membrane area available for diffusion is fixed. (The dialyser block design is discussed in ref. 13.) The factors that can influence dialysis efficiency are thus limited to dialysis time and to factors influencing either the diffusity of the solute or the concentration gradient. The diffusivity is inversely related to the viscosity of the donor medium [12], and cuprophane membrane resistances are shown to depend linearly on molecular mass for many solutes [14]. A steep concentration gradient across the membrane is important to get a large mass transfer. This can be accomplished by releasing any bound solute from the matrix and by supplying fresh donor and acceptor solutions continuously or in pulses.

*Method development*

In the development of a method for determination of OA and FQ, some of the factors mentioned above were optimized or evaluated. Using a dialysis time of 7.3 min the ASTED cycle was just finished within the HPLC analysis time of the previous sample.

As the samples analysed are quite viscous, it was interesting to investigate if their viscosity influences the dialysis efficiency. Using dextran to prepare solutions of various viscosities, and a capillary viscosimeter to measure viscosity, no decrease in dialysis efficiency could be observed for OA, NA or FQ when the relative viscosity of the donor solution was varied from 1 (distilled water) to more than 3. By comparison, the viscosity of the donor solution was 1.7 when plasma samples were analysed. Accordingly, under the conditions used, the viscosity of the donor solution did not significantly influence the dialysis efficiency in the range of viscosities investigated, not obeying the equations reviewed in ref. 12.

The molecules of OA, NA and FQ are highly bound to proteins. Turnell and Cooper [6] showed that trichloroacetic acid (TCA) in a buffered solution displaces phenytoin from binding sites on serum proteins by competition, increasing the dialysis efficiency and lowering the between-sample imprecision [7]. To release OA, NA and FQ from proteins, monochloroacetic acid and TCA in buffered solutions (pH 7) were examined. TCA proved to give the highest dialysis efficiencies, and the concentration of TCA in the internal standard reagent was optimized to a concentration of 0.2 *M* (Fig. 2). The concentration of TCA was varied by changing the ratio of 1 *M* TCA to 1 *M* phosphate buffer used to dilute the internal standard. The dilution of the samples with internal standard solution *per se* also causes the drugs to be released from the proteins [15].

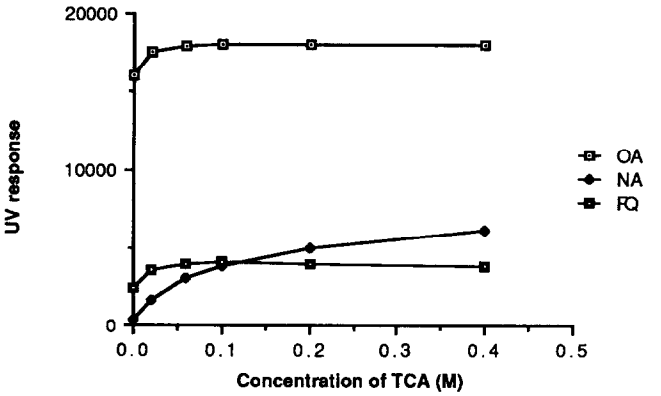


Fig. 2. UV response (peak heights) of OA, NA and FQ *versus* concentration of TCA.

The recipient solution was passed through the dialyser in pulses. Fig. 3 shows the dialysis efficiencies of OA, NA and FQ in plasma and whole blood when the volume of the recipient solution was varied from 1 to 8 ml and the dialysis time was kept at 7.3 min. Efficiencies were calculated by relating the peak heights to the peak height obtained when an aqueous solution of the drugs was injected directly into the trace enrichment column. With 4 ml of recipient solution, the dialysis efficiencies were in the range 60–69%. The volume of 4 ml was chosen so as to limit the amounts of trace impurities, present in the recipient solution that passed into the highly hydrophobic precolumn; the gain in efficiency from using 8 ml of recipient solution was very small. Pulsation of the recipient solution is especially useful if the analytes readily break through the enrichment column. This is not a problem in this case; even when 32 ml of recipient solution were

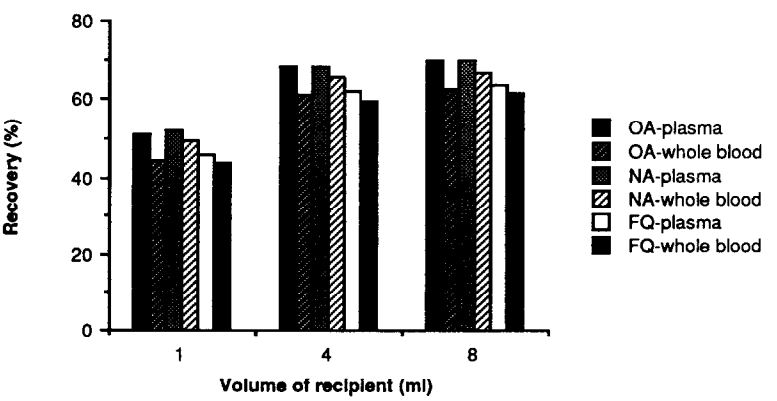


Fig. 3. Recovery of OA, NA and FQ in salmon plasma and whole blood *versus* volume of recipient solution. In all instances dialysis time was 7.3 min.

pumped through this column, no break-through was observed. Stegehuis *et al.* [16] pointed out that, if a significant difference in ionic strength exists between the donor and acceptor streams, the result is a flow of water through the membrane, which produces pressure irregularities that influence the mass flow across the membrane. In the present method such a difference exists because of the conditions used to break the protein bonding of the drugs, but despite this the dialysis efficiencies are satisfactorily high.

*Limit of detection*

The limit of detection in whole blood and plasma was determined to be 50 ng/ml for both OA and FQ, at a detection wavelength of 325 nm and a signal-to-noise ratio of 3. The limit could have been further lowered by using a fluorescence detector, or, if large volumes of samples were available, a larger dialysis cell, two cells connected in series or several portions of sample dialysed. Trace-level determination of nitrofuranes was accomplished by dialysing several portions of sample [14], but at the expense of a long dialysis time. However, for the purpose of pharmacokinetic studies of OA and FQ in salmon, a lower limit of detection of 50 ng/ml is considered satisfactory.

*Assay validation*

Analyte concentrations were determined from their peak-height ratios relative to the internal standard, using calibration standards made from the same biological material as the samples. The calibration standards (0.2, 3.0, 6.0 and 10.0 μg/ml) gave correlation coefficients of 0.9999. The results in Table I show that OA

TABLE I

WITHIN-DAY ACCURACY AND PRECISION

P = plasma; WB = whole blood; $n$ = 6.

| Analyte | Matrix | Concentration added (μg/ml) | Concentration found (μg/ml) | C.V. (%) | Error (%) |
|---|---|---|---|---|---|
| OA | P | 0.50 | 0.52 | 3.1 | 4.0 |
| | WB | 0.50 | 0.54 | 2.0 | 8.0 |
| | P | 2.00 | 2.05 | 1.1 | 2.5 |
| | WB | 2.00 | 2.07 | 0.9 | 3.5 |
| | P | 5.00 | 5.00 | 1.5 | 0 |
| | WB | 5.00 | 5.06 | 1.3 | 1.2 |
| FQ | P | 0.50 | 0.51 | 5.3 | 2.0 |
| | WB | 0.50 | 0.53 | 3.7 | 6.0 |
| | P | 2.00 | 2.05 | 0.6 | 2.5 |
| | WB | 2.00 | 2.05 | 0.8 | 2.5 |
| | P | 5.00 | 5.01 | 1.3 | 0.2 |
| | WB | 5.00 | 5.09 | 1.2 | 1.8 |

TABLE II

BETWEEN-DAY ACCURACY AND PRECISION

P = plasma; WB = whole blood; $n = 6$.

| Analyte | Matrix | Concentration added (μg/ml) | Concentration found (μg/ml) | C.V. (%) | Error (%) |
|---|---|---|---|---|---|
| OA | P | 2.00 | 1.95 | 3.0 | 2.5 |
| | WB | 2.00 | 1.98 | 2.5 | 1.0 |
| | P | 5.00 | 4.94 | 2.0 | 1.2 |
| | WB | 5.00 | 5.11 | 2.4 | 2.2 |
| FQ | P | 2.00 | 1.89 | 4.6 | 5.5 |
| | WB | 2.00 | 2.03 | 3.8 | 1.5 |
| | P | 5.00 | 4.86 | 1.7 | 2.8 |
| | WB | 5.00 | 5.04 | 2.0 | 0.8 |

and FQ are determined with good precision and accuracy, both in plasma and whole blood.

The between-day variation was determined by analysing one parallel of 2.0 and 5.0 μg/ml standards on six consecutive days, storing them at 4°C during the week. The results are shown in Table II. The accuracy is as for samples analysed on the same day (Table I), and the coefficients of variation (C.V.) are slightly higher.

The results obtained in whole blood were compared with those obtained in plasma, using linear regression. For this experiment, standards were prepared in fresh blood, and plasma standards were prepared by centrifugation from all these standards. From standards of 0.5, 2.0 and 5.0 μg/ml, six parallel samples were analysed from the whole blood and the corresponding plasma standards. Whole blood values ($y$) were plotted *versus* the plasma values ($x$) for both OA and FQ. The correlation coefficients obtained in this way were better than 0.999, and the equations for the regression lines were $y = -0.10 + 1.05x$ and $y = -0.05 + 1.01x$ for oxolinic acid and flumequine, respectively. This shows that the correlation between the results obtained in plasma and those obtained in whole blood is good.

## CONCLUSION

Using the combination of dialysis, trace enrichment and a simple HPLC system, oxolinic acid and flumequine can be determined with high precision; the dialysis process gives high and reproducible recoveries. The method is easy to carry out; no manual treatment of the samples is necessary. Samples can be prepared and analysed at the rate of 4.4 per hour and, as the ASTED system can be run without human intervention, the capacity of the system is high. The sim-

plicity of the method, the high sample throughput, the detection limit of 50 ng/ml and the small sample volume required all make the method well suited for pharmacokinetic studies in salmon.

ACKNOWLEDGEMENTS

This work has been financially supported by the Norwegian Research Council for Science and the Humanities and by Norsk Medisinaldepot (Oslo, Norway).

REFERENCES

1 K. Hamamoto, *J. Chromatogr.*, 381 (1986) 453.
2 D. Decolin, A. Nicolas and G. Siest, *J. Chromatogr.*, 414 (1987) 499.
3 L. Ellerbroek and M. Bruhn, *J. Chromatogr.*, 495 (1989) 314.
4 S. O. Hustvedt, R. Salte and T. Benjaminsen, *J. Chromatogr.*, 494 (1989) 335.
5 K. E. Rasmussen, F. Tønnesen, T. H. Hoang, A. Rogstad and A. Aanesrud, *J. Chromatogr.*, 496 (1989) 355.
6 D. C. Turnell and J. D. H. Cooper, *J. Chromatogr.*, 395 (1987) 613.
7 J. D. H. Cooper, D. C. Turnell, B. Green and F. Verillon, *J. Chromatogr.*, 456 (1988) 53.
8 T. H. Hoang, A. T. Andresen, T. Agasøster and K. E. Rasmussen, *J. Chromatogr.*, 532 (1990) 363.
9 A. Rogstad, V. Hormazabal and M. Yndestad, *J. Liq. Chromatogr.*, 12 (1989) 3073.
10 K. Eivindvik and K. E. Rasmussen, *J. Liq. Chromatogr.*, 12 (1989) 3061.
11 O. B. Samuelsen, *J. Chromatogr.*, 497 (1989) 355.
12 G. L. Flynn, S. H. Yalkowsky and T. J. Roseman, *J. Pharm. Sci.*, 63 (1974) 479.
13 M. M. L. Aerts, W. M. J. Beek and U. A. Th. Brinkman, *J. Chromatogr.*, 500 (1990) 453.
14 P. C. Farrell and A. L. Babb, *J. Biomed. Mater. Res.*, 7 (1973) 275.
15 R. D. McDowall, *J. Chromatogr.*, 492 (1989) 3.
16 D. S. Stegehuis, U. R. Tjaden and J. van den Greef, *J. Chromatogr.*, 511 (1990) 137.