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Fully automated high-performance liquid chromatographic analysis of whole blood and plasma samples using on-line dialysis as sample preparation

Determination of oxytetracycline in bovine and salmon whole blood and plasma

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ABSTRACT

A fully automated technique for high-performance liquid chromatographic analysis of whole blood and plasma is described. Samples are automatically injected into a dialyser where proteins and blood cells are removed. The dialysates are concentrated on a small column prior to analysis. This technique is used for the determination of oxytetracycline in whole blood and plasma. After dialysis oxytetracycline and the internal standard, tetracycline, are retained on a polystyrene enrichment column and subsequently separated on a polystyrene analytical column by ion-pair chromatography. Using ultraviolet detection 50 ng/ml can be detected. Validation showed good within-day and between-day accuracy and precision. Different oxytetracycline concentrations were found in plasma and whole blood. This difference varied between the species.

INTRODUCTION

When monitoring drugs in blood direct determination in whole blood may be required. For example, in pharmacokinetic studies of small animals (*e.g.* fish) containing small amounts of blood, analysis of whole blood is advantageous compared to analysis of plasma or serum, because much of the sample is wasted during plasma or serum preparation. Whole blood analysis makes full use of the sample. In field studies carried out far from the laboratory it is practical to freeze the blood samples directly, without having to prepare plasma or serum on the spot. In such cases methods for analysing haemolysed whole blood are necessary. Analysis of whole blood may also be favourable in other situations, in order to avoid plasma or serum preparation. For the interpretation of the results the correlation between plasma and whole blood concentrations need to be established.

Until now analysis of blood samples by high-performance liquid chromatogra-

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phy (HPLC) has been complicated by time-consuming work-up procedures. The work-up included protein precipitation, liquid–liquid extraction or solid-phase extraction. However, using dialysis as a purification step prior to HPLC, whole blood analysis is considerably simplified and fully automated methods can be developed [1,2]. Crude blood samples (haemolysed or not) are injected on the upper side of a dialysis membrane. Only low-molecular-mass compounds traverse the membrane while proteins and particles (*e.g.* cellular components) are removed. Using an enrichment column after the dialysis cell, dialysis and HPLC can be connected on-line. This is the principle of the fully automated ASTED (automated sequential trace enrichment of dialysates) system. Dialysis efficiencies of up to 85% in 3 min have been reported for aqueous nitrofurane solutions [3]. Addition of releasing agents may increase the dialysis efficiencies of protein-bound drugs in biological samples. Dialysis efficiencies of 60–70% in 7 min have been obtained in plasma and whole blood [1]. Efficiencies of this order are also obtained in milk [4] and tissue extracts [5].

This paper describes a method for the analysis of oxytetracycline (OTC) in bovine and salmon whole blood and plasma. The fully automated procedure is based on the ASTED system connected on-line with an HPLC system. OTC is widely used in human and veterinary medicine including aquaculture. Field studies in aquaculture have created a demand for fully automated methods for whole blood analysis. Several methods for the determination of OTC in plasma or serum have been published [6–13], but no method for OTC in whole blood seems to be available.

EXPERIMENTAL

Chemicals and reagents

Oxytetracycline hydrochloride was obtained from Norsk Medisinaldepot (Oslo, Norway) and hydrochlorides of tetracycline (TC), doxycycline (DOX), demeclocycline (DMC) and minocycline (MC) were obtained from Sigma (St. Louis, MO, USA). Sodium dihydrogenphosphate, orthophosphoric acid and Triton X-100 were obtained from Merck (Darmstadt, Germany), 1-heptanesulphonic acid (sodium salt, monohydrate) from Fluka (Buchs, Switzerland) and sodium hydroxide from EKA (Bohus, Sweden). HPLC-grade acetonitrile was from May & Baker (Dagenham, UK). Water was purified with a Milli-Q system (Millipore, Milford, MA, USA).

Preparation of standards

Stock solutions of OTC and TC (1 mg/ml) were prepared in methanol–water (1:1, v/v). These solutions were stored at −20°C for up to one month. Working standards of OTC (25 μg/ml) and internal standard (100 μg/ml) were prepared by dilution with water. Citrated bovine blood and heparinised salmon blood were used. Bovine blood was used for method development and validation because of

its ready availability. Plasma was prepared by centrifuging the blood 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 OTC working standard. The internal standard solution used in this study was 6 μg/ml TC in water.

Apparatus

A schematic representation of the apparatus is shown in Fig. 1. The ASTED system (Gilson Medical Electronics, Villiers-le-Bel, France) comprised a sample injector (Model 231) and two dilutors (Model 401) equipped with 1-ml syringes, plus a dialysis cell with a donor volume of 100 μl fitted with a cellulose dialysis membrane (Cuprophan) with a molecular mass cut-off of 15 kDa. A column (10 mm × 2.0 mm I.D.) (Chrompack, Middelburg, Netherlands) packed with 36 μm polystyrene (Dynospheres, Dyno Particles, Lillestrøm, Norway) was mounted on a Model 7010 six-port valve (Rheodyne, Berkeley, CA USA) that connected the trace enrichment column to the recipient channel of the dialysis cell or to the HPLC system when the valve was switched. The priming solution for the donor side of the system was 0.01% (w/v) Triton X-100 in water. The recipient solution was 0.02 *M* phosphate buffer pH 5, containing 0.005 *M* heptanesulphonic acid, sodium salt.

The chromatographic system consisted of an LC-6A isocratic pump (Shimadzu, Kyoto, Japan), an SPD-6AV UV–visible spectrophotometric detector (Shimadzu) set at a wavelength of 350 nm, a polystyrene analytical column (PLRP-S, particle size 5 μm, 150 mm × 4.6 mm I.D.) (Polymer Labs., Church Stretton, UK) and a Chromatopac C-R3A integrator (Shimadzu). The mobile phase, acetonitrile–0.005 *M* heptanesulphonic acid (sodium salt), 0.02 *M* orthophosphoric acid (23:77, v/v) was pumped at a flow-rate of 0.7 ml/min.

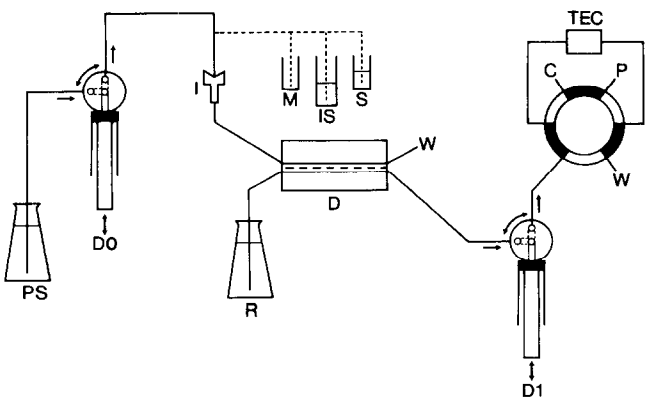

Fig. 1. Schematic representation of the combined ASTED and HPLC systems. PS = priming solution; D0 = dilutor 0; S = sample; IS = internal standard; M = mixing vial; I = injector; D = dialyser; R = recipient solution; W = waste; D1 = dilutor 1; TEC = trace enrichment column; P = HPLC pump; C = column.

ASTED sample preparation procedure

Through dilutor 0 (Fig. 1) 125 μl of sample were mixed with 25 μl of internal standard solution, and the donor channel of 100 μl was overfilled with this mixture. There it was kept static for 7.3 min while 4 ml of recipient solution were pumped through the recipient channel in pulses by dilutor 1 (Fig. 1), 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, dilutor 1 being programmed to pump 1.7 ml/min, and keeping them static for *ca.* 13 s.

Subsequently OTC and the internal standard were eluted to the analytical column by back-flushing with HPLC mobile phase for 2 min. At the same time, residual sample and dialysate were purged out of the dialyser by the two dilutors, using 2 ml of the respective solutions. Following elution the trace enrichment column was regenerated with 2 ml of recipient solution. In the concurrent operation of sample preparation and analysis, samples were prepared during the 13-min analysis of the previous sample.

RESULTS AND DISCUSSION

HPLC separation

In recent years polystyrene columns have been preferred to silica-based phases for tetracyclines by many authors [6,9,11,14–18]. This includes methods for purity control where tetracyclines are separated from closely related products formed during production and degradation. Acidic mobile phases containing anions from sulphonic acids have previously been described for OTC separations on C_{18} [12] and NH_2 [19] columns. In the present method OTC and the internal standard are retained on a polystyrene column as ion pairs of 1-heptanesulphonic acid. Four other tetracyclines, TC, DOX, DMC and MC, were investigated as internal standards for OTC. TC [8] and DMC [20] have previously been used as internal standards for OTC. TC appeared to be the best choice in this system. The chromatograms in Fig. 2 show baseline separation of OTC and TC.

Trace enrichment

In a previously published column-switching method for the determination of OTC in plasma [6] the same polystyrene precolumn as the one used as a trace enrichment column in the present method was used. Using phosphate buffer at an optimum pH of 3.5 break-through of OTC occurred at eluent volumes larger than 0.75 ml.

When dialysis is used as sample preparation larger volumes than 0.75 ml need to be transported through the dialysis cell to give high recoveries in a short time. To achieve this the anion of 1-heptanesulphonic acid was added to the recipient solution at a concentration of 0.005 *M*. The OTC and TC ion pairs were very well retained on the trace enrichment column. No break-through could be observed,

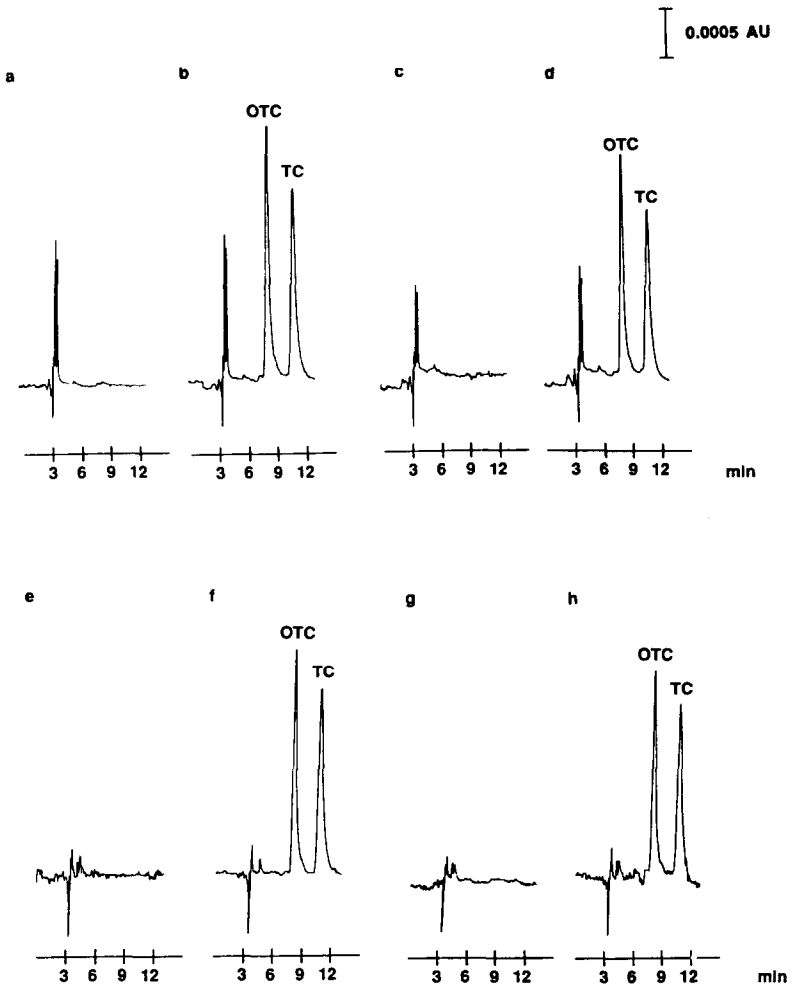

Fig. 2. Chromatograms after dialysis of (a) drug-free salmon plasma, (b) salmon plasma spiked with 1 μg/ml OTC, (c) drug-free salmon whole blood, (d) salmon whole blood spiked with 1 μg/ml OTC, (e) drug-free bovine plasma, (f) bovine plasma spiked with 1 μg/ml OTC, (g) drug-free bovine whole blood and (h) bovine whole blood spiked with 1 μg/ml OTC. For (a), (c), (e) and (g), 25 μl of water were added, while for (b), (d), (f) and (h) 25 μl of TC internal standard were added.

even when 32 ml of recipient solution were passed through the column. This holds true when the recipient solution is acidic; at a pH of 7.0 recovery decreased slightly when 16 ml or more were passed through. A recipient solution of pH 5.0 was chosen. Recipient volumes which have previously been used are in the range 2–9 ml.

Dialysis

Only molecules that are not bound to proteins are able to traverse the membrane. TC is 24–65% bound to plasma proteins and OTC is 20–35% bound to serum proteins [21]. The effect of addition of various reagents on protein binding was investigated. The reagents investigated were EDTA (0.1 *M*), heptanesulphonic acid (sodium salt, 0.03 *M*) and phosphate-buffered (pH 7.0, 0.6 *M*) trichloroacetic acid (TCA) (0.2 *M*), respectively. Buffered TCA (pH 7) has previously been used to release other drugs from proteins [1,2,22]. None of these reagents were successful in increasing the dialysis efficiencies, and therefore an aqueous solution of the internal standard was added to the sample prior to dialysis.

The recoveries of OTC and TC were investigated as a function of recipient volume passed through the recipient channel of the dialyser. Recoveries were calculated by relating the peak heights to the peak heights obtained when an aqueous solution of the drugs was injected directly into the trace enrichment column. The results are shown in Fig. 3. As the recoveries did not increase significantly from 4 to 8 ml, 4 ml of recipient solution was chosen. The recoveries are of the same order as previously obtained for oxolinic acid and flumequine [1], and they are somewhat lower in whole blood than in plasma. It has previously been shown that sample viscosity does not influence dialysis efficiency [1], and it is likely that the difference in recovery from whole blood and plasma is due to binding of the drugs to blood cells or that the blood cells limit the diffusion of OTC and TC through the donor medium. The chromatograms in Fig. 2 show that the combined dialysis and enrichment effectively removes the large fronts that are often associated with plasma and serum injections. The bovine and salmon whole blood and plasma blanks do not contain substances that would interfere with OTC or TC detection. Chromatograms obtained after dialysis of bovine and salmon whole blood and plasma spiked with 1 μg/ml OTC are also shown in Fig. 2.

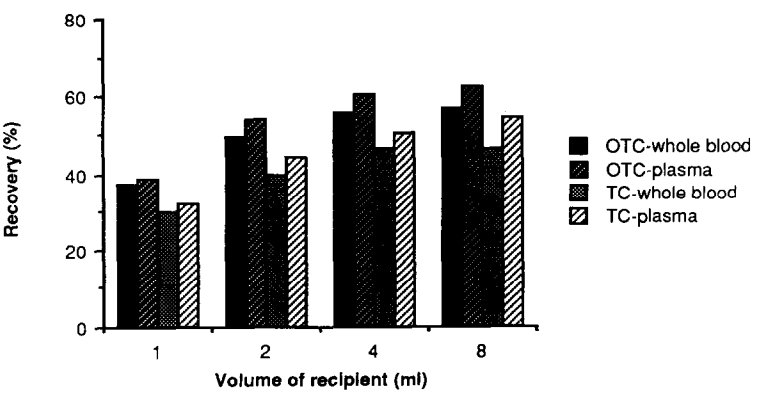

Fig. 3. Recovery of OTC and TC in bovine plasma and whole blood *versus* volume of recipient solution. In all instances the dialysis time was 7.3 min.

Limit of detection

Concentrations of OTC and TC as low as 50 ng/ml could be detected at a signal-to-noise ratio of 3.

Stability

The stability of OTC plasma and whole blood samples and TC internal standard solution at room temperature was investigated. During a period of 24 h no decrease in concentration occurred. The working standards of OTC and TC could be stored at 4°C for at least one week without degradation.

The stability of the dialysis membrane, the enrichment column and the HPLC column were also good. The dialysis membrane and the enrichment column could be used for *ca.* 1000 samples. In our laboratory the analytical column has been used in this system and related systems for three years.

Assay validation

OTC concentrations were determined from the OTC/internal standard peak-height ratios, using calibration standards made from blood when blood samples were analysed, or from plasma when plasma samples were analysed. The calibration standards (0.2, 1.5, 3.0 and 5.0 μg/ml) gave correlation coefficients of 0.9998 or better. Within-day and between-day accuracy and precision were determined. Samples for the between-day study were kept at −20°C until analysis. The results are shown in Table I.

Correlation between whole blood and plasma determination

Whole blood concentrations were determined from a standard curve made by spiking whole blood. When plasma was produced from these whole blood standards, a corresponding standard curve could be made for plasma. This standard

TABLE I

WITHIN-DAY AND BETWEEN-DAY ACCURACY AND PRECISION (n = 6)

Concentration added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)		C.V. (%)	
	Within-day	Between-day	Within-day	Between-day
Plasma				
0.50	0.48 ± 0.02	0.45 ± 0.02	3.9	3.5
1.50	1.49 ± 0.04	1.52 ± 0.03	2.5	2.1
5.00	4.99 ± 0.06	5.09 ± 0.14	1.2	2.8
Whole blood				
0.50	0.52 ± 0.02	0.52 ± 0.01	4.7	2.3
1.50	1.49 ± 0.03	1.54 ± 0.03	2.1	2.0
5.00	5.02 ± 0.08	5.12 ± 0.10	1.6	1.9

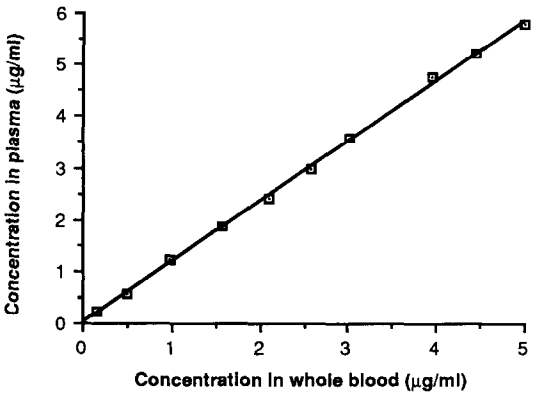

Fig. 4. OTC concentrations in whole blood *versus* actual plasma concentrations (bovine blood).

curve could be used to calculate whole blood concentrations after analysis of plasma samples. This was verified by the following experiment. Ten samples of fresh bovine blood of different concentrations of OTC (0.2–0.5 µg/ml) and calibration standards were prepared. The remaining blood spiked with OTC was centrifuged and ten plasma samples of different concentration and calibration standards were taken. After preparation the samples were stored at −20°C until analysis. The whole blood and plasma samples gave the same results when their corresponding standard curves were used, as shown by the equation $y = -0.01 + 1.01x$, $r = 0.9994$ where x represents the results in whole blood and y the results in plasma.

The actual plasma concentrations are found from a standard curve made by spiking plasma. For bovine plasma this standard curve, when used for the above plasma samples, gave *ca.* 20% higher plasma concentrations than whole blood concentrations as shown by the equation $y = 0.02 + 1.17x$, $r = 0.9994$ where x is the whole blood concentration and y is the plasma concentrations (Fig. 4).

Whole blood and plasma concentrations may differ for many drugs. Drugs which are extensively bound to blood cells show lower plasma than whole blood concentrations. (Blood cells are removed during plasma preparation.) Drugs which are not (or only to a small degree) bound to or distributed to blood cells show higher plasma than whole blood concentrations. Identical whole blood and plasma concentrations can only be obtained if a drug is evenly distributed between blood cells and plasma. The results obtained in bovine blood suggest that OTC is not much bound to blood cells. In a similar experiment that was carried out in salmon blood the results were *ca.* 10% lower in plasma than in whole blood. This suggests more binding or more distribution of OTC to salmon blood cells than to bovine blood cells. Tetracyclines have been shown to bind to phospholipids [23] and suggested to be partly bound to cellular components of the blood [24]. It is not unlikely that such binding conditions may vary between

species. The present on-line combination of dialysis and HPLC is a promising technique for carrying out such correlation studies.

CONCLUSION

Using the ASTED system whole blood samples were analysed just as readily as plasma samples, making the preparation of plasma unnecessary in many cases. Correlations between drug concentrations in whole blood and plasma could easily be found. The simplicity of the method (no manual sample work-up), the high sample throughput (4.6 samples per hour), the low detection limit and the small sample volume required all made the method well suited for drug analysis in whole blood and plasma.

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