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RAPID QUANTITATIVE DETERMINATION OF SEVEN ANTHRACYCLINES AND THEIR HYDROXY METABOLITES IN BODY FLUIDS

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the determination of seven anthracycline analogues and their hydroxy metabolites. The method is suitable for analysis of 30 plasma samples a day. The detection limit is 0.5 ng/ml for all compounds. Examples of the pharmacokinetics of adriamycin and carminomycin in man are shown.

INTRODUCTION

Since the introduction of the first anthracycline, daunorubicine, in clinical practice for the treatment of cancer [1], a number of analytical methods have been designed to determine concentrations of these compounds and their metabolites in plasma, urine, bile and in the intracellular fluid of different kinds of tissue.

The methods, which have recently been summarized by Arcamone [2], include fluorometry [3, 4], thin-layer chromatography [5, 6], high-performance liquid chromatography [7—13] and methods in which radioactively labeled anthracyclines are used, such as radioimmunoassay (^{3}H) [14] and total body autoradiography (^{14}C) [15].

The analytical procedure described in this paper is a modification of the reversed-phase method of Eksborg et al. [10], permitting the determination of seven anthracyclines: adriamycin (ADM), daunorubicin (DAR), carminomycin (CAM), 4′-epi-adriamycin (4′-epi-ADM), all in clinical use and of the experimental drugs 4-demethoxy-adriamycin (4-dem-ADM), 4′-deoxy-adriamycin (4′-deo-ADM), and 4-demethoxy-daunorubicin (4-dem-DAR) (Fig. 1).

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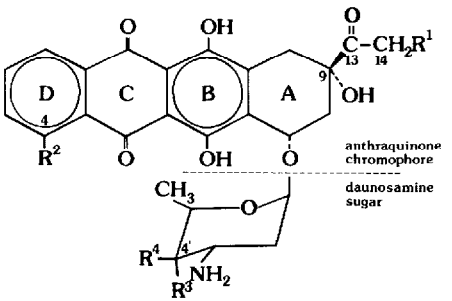

Fig. 1. Structural variations of seven anthracycline analogues.

	R^1	R^2	R^3	R^4
ADM	OH	OCH_3	OH	H
4'-epi-ADM	OH	OCH_3	H	OH
4'-deo-ADM	OH	OCH_3	H	H
4-dem-ADM	OH	H	OH	H
DAR	H	OCH_3	OH	H
CAM	H	OH	OH	H
4-dem-DAR	H	H	OH	H

The active 13-hydroxy metabolites can be determined in the same run. No other active metabolites have yet been reported [5].

The procedure was developed to obtain a determination method which could be easily performed, allowing analysis of 30 plasma samples a day and which would also lower the detection limit. The method is based on an on-column concentration and cleaning procedure [16], which allows injection of large volumes and thus lowers the detection limit. The method has been applied to pharmacokinetic studies performed in humans and dogs. Some examples of the pharmacokinetic behaviour of ADM and CAM and their hydroxy metabolites in man are shown.

MATERIALS AND METHODS

Drugs and chemicals

4-dem-DAR, 4-dem-ADM, 4'-deo-ADM and adriamycinol (ADM-ol) were kindly supplied by Farmitalia (Milan, Italy) and desipramine by Ciba-Geigy (Arnhem, The Netherlands). All other drugs were commercially available for clinical application and were obtained from: Roger Bellon (Neuilly, France) ADM; Specia (Paris, France) DAR; Bristol Meyers (Syracuse, NY, U.S.A.) CAM; Farmitalia (Milan, Italy) 4'-epi-ADM. All chemicals were of analytical grade and were used without further purification.

Chromatography

The chromatographic system consisted of a double-head solvent pump (Orlita, DHP-1515, Bakkers & Co., Zwijndrecht, The Netherlands), two sampling valves (Valco, Houston, TX, U.S.A.) and a sampling loop of 1 ml. Complete pulse quenching was achieved by means of a Silica Si 60 column (25 cm × 4.6 mm I.D.), followed by 2 m of coiled tubing (4 cm I.D.), between

the pump and injection valve. The analytical column (15 cm × 4.6 mm I.D.) was packed with reversed-phase material Cp^{tm} Spher C8, particle size 8 μm (Chrompack, Middelburg, The Netherlands). The concentration column (5 cm × 3.0 mm I.D.) was filled with LiChrosorb RP-8, 10 μm (Chrompack).

Detection

A fluorescence detector (Perkin-Elmer, Model 3000, Delft, The Netherlands), equipped with a red sensitive photomultiplier and a double mirrored flow cuvette was used. The maximum excitation wavelength was 474 nm. Alterations in the electron density of the D-ring (Fig. 1) shift the maximum emission wavelength from 590 nm to 568 nm for 4-dem-DAR and 4-dem-ADM and to 555 nm for CAM. The detector was connected to a 10-mV recorder (BD 40, Kipp & Zonen, Delft, The Netherlands). All experiments were carried out at room temperature.

Cleaning procedure and enrichment method [16]

The sample loop of 1 ml was filled as shown in Fig. 2b. By turning the upper valve, solvent A transports the sample onto the concentration column (Fig. 2a), where the anthracyclines and hydroxy metabolites are retained and concentrated while those products with more hydrophilic properties are wasted. By turning the lower valve, solvent B will flush the concentrated sample onto the analytical column (Fig. 2b). To obtain a sufficient clean up of the sample, 7 ml of solvent A was used. The sample could then be transported with 3 ml of solvent B from the concentration column onto the analytical column.

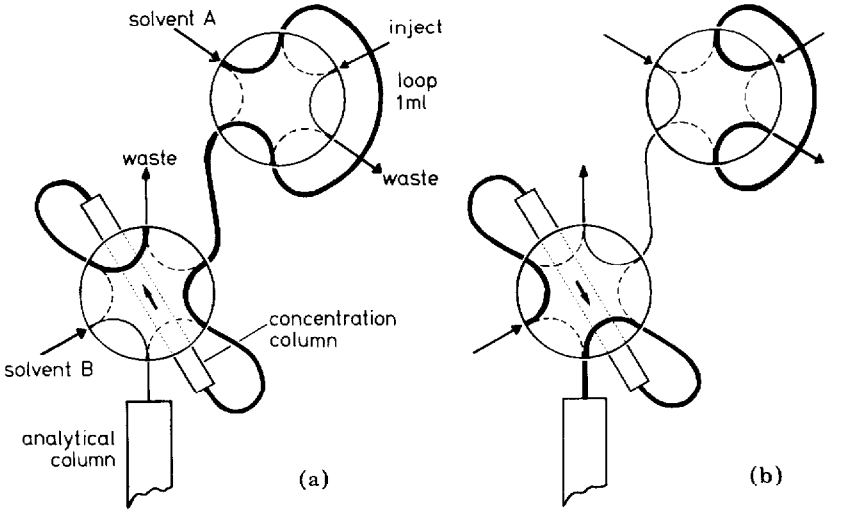

Fig. 2. Diagram showing sample enrichment through the on-column concentration method.

Solvents

Solvent A consisted of a 10 μg/ml solution of desipramine in demineralized water. Desipramine is added to limit the absorption of the anthracyclines

and their hydroxy metabolites on the tubing and column. The liquid flow-rate was kept between 1.5 and 2.0 ml/min. Solvent B consisted of a mixture of acetonitrile—citric acid buffer (9.8 ml of 0.1 *M* citric acid + 0.2 ml of 0.2 *M* Na_2HPO_4, pH 2.2)—demineralized water [x:10:(90—x), v/v]. All anthracyclines could be measured in the same retention time area (± 7 min) by slight alterations in the acetonitrile concentration : ADM and 4'-epi-ADM, 30% v/v; 4-dem-ADM and 4'-deo-ADM, 35% v/v; DAR, 40% v/v; CAM and 4-dem-DAR, 45% v/v. The liquid flow-rate was 1.0 ml/min at a pressure of 5 MPa.

Sample preparation

Plasma. Using a polypropylene tube to avoid absorption, 1.0 ml of boric acid buffer (pH 8.9) and 5.0 ml of a mixture of chloroform—isopropanol (4:1) were added to 1.0 ml of plasma. After mixing for 1 min on a Vortex mixer followed by centrifugation at 1400 *g*, the lower organic layer was transferred into another polypropylene tube. Then 1.0 ml of 0.1 *M* H_3PO_4 was added and the resulting mixture was mixed on a Vortex mixer for 30 sec. After centrifugation, 1.0 ml of the aqueous layer was injected into the chromatographic system.

Urine. Urine samples were diluted with 0.1 *M* H_3PO_4 according to the anticipated concentration and injected without further extractions.

Recovery

The recoveries of parent compounds in plasma were measured in triplicate over a concentration range from 5 to 500 ng/ml and were compared to the direct assay of the standards in 0.1 *M* H_3PO_4. The hydroxy metabolites are not available as pure substances, but can be found in urine after administration of the parent compounds. By dividing the areas under the peaks of the chromatogram for urine, which can be injected without extraction, by those areas obtained from the extracted blank plasma that was spiked with a small predetermined quantity of the same urine (containing a high concentration of the metabolite), the recovery for the extraction procedure of the metabolites could be determined (Table I).

Stability

To study whether the handling and storage of the plasma samples had an influence on the plasma concentration of the anthracyclines, spiked plasma containing 100 ng/ml ADM and ADM-ol was kept at —20°C. During seven consecutive days this plasma was thawed, the concentration of ADM and ADM-ol determined and refrozen again. Also samples which were stored at —20°C during fourteen days were analyzed.

Calibration curve

Using spiked plasma, a calibration curve in the anticipated concentration range (from 5 to 500 ng/ml) was made each morning before analysis. During the day, the analytical equipment was checked by injection of spiked plasma. The correlation coefficient of the calibration curves were always better than 0.98.

Pharmacokinetics

An example of the application of this method was the study of the pharmacokinetics of ADM and CAM. In two patients, admitted to the Sint Radboud Hospital (Nijmegen, The Netherlands) for treatment of their advanced malig-

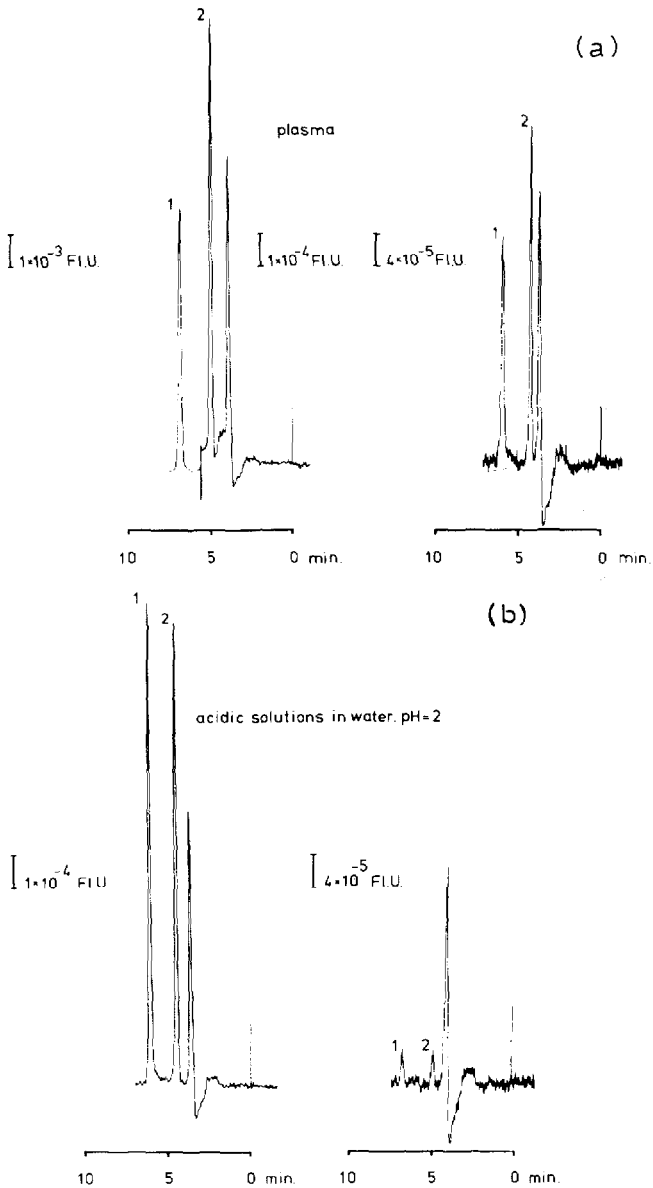

Fig. 3. (a) Chromatograms of plasma samples of a patient receiving 55 mg/m² body surface ADM by i.v. bolus injection. At 0.5 h after the administration, the plasma concentration of ADM (1) was 125 ng/ml and of ADM-ol (2) 19 ng/ml (left). Values of 5.9 ng/ml ADM (1) and 5.3 ng/ml ADM-ol (2) were measured 66 h after administration (right). (b) Chromatogram of an acidic aqueous solution of 20 ng/ml ADM (1) and 13.4 ng/ml ADM-ol (2) (left). The detection limit at a signal-to-noise ratio of 3 was 0.5 ng/ml for ADM (1) and 0.4 ng/ml for ADM-ol (2) (right).

nant disease after an intravenous (i.v.) bolus injection of 1.22 mg/kg ADM and of 0.36 mg/kg CAM. There was evidence of normal renal, hepatic and bone marrow function and no active cardiac disease. The patients gave informed consent. Blood samples were obtained at regular time intervals via an indwelling venous catheter and collected in heparinized tubes. Urine samples were taken from spontaneously voided urine.

The plasma concentration data were fitted according to a three-compartment open model, and the pharmacokinetic parameters were calculated according to conventional procedures [17].

RESULTS

Chromatography

Chromatograms of plasma samples obtained after 0.5 and 66 h from a patient, treated with an i.v. bolus dose of adriamycin of 55 mg/m^2 body surface are shown in Fig. 3a. Blank plasma showed only the injection peak. In Fig. 3b, the chromatograms of the acidic aqueous solutions in water are given, showing the detection limit of 0.5 ng/ml for ADM and of 0.4 ng/ml for ADM-ol at a signal-to-noise ratio of 3. The other anthracyclines all show the same pattern with different acetonitrile concentrations. The capacity ratio's (k') measured with an acetonitrile concentration of 35% v/v of the seven anthracyclines and their hydroxy metabolites are listed in Table I. The covariance in the concentration determinations of the anthracyclines and their hydroxy metabolites calculated from five spiked plasma samples were better than 5% in the concentration range of 5 to 500 ng/ml. The recovery rates after the extraction procedure of the seven analogues and their hydroxy metabolites are listed in Table I.

TABLE I

CAPACITY RATIOS (k') AND RECOVERY PERCENTAGES

For description of analytical procedures, see text.

Compound	k' (35% acetonitrile)	Recovery (%) (mean ± S.D.)
ADM	0.55	89 ± 2
ADM-ol	0.15	81 ± 3
4'-epi-ADM	0.74	87 ± 2
4'-epi-ADM-ol	0.29	79 ± 4
4-dem-ADM	1.02	90 ± 2
4-dem-ADM-ol	0.46	81 ± 3
4'-deo-ADM	1.28	93 ± 2
4'-deo-ADM-ol	0.53	83 ± 3
DAR	1.77	82 ± 2
DAR-ol	0.76	90 ± 3
CAM	2.98	74 ± 2
CAM-ol	1.50	83 ± 1
4-dem-DAR	2.98	80 ± 3
4-dem-DAR-ol	1.50	90 ± 1

Stability

During the seven consecutive days of thawing and refreezing, no decrease in the plasma concentrations of ADM and ADM-ol was observed. Also the concentrations of the samples after a storage period of fourteen days were found to be unchanged.

Clinical pharmacokinetics

Figs. 4 and 5 show the plasma concentration—time and renal excretion rate—time profiles of the parent compounds and their hydroxy metabolites in two patients after an i.v. bolus injection of ADM and CAM, respectively. Only 10% of the dosage administered is recovered in the urine as unchanged drug. A large difference exists between the percentage of the metabolites recovered in the urine (2.2% ADM-ol; 66% CAM-ol).

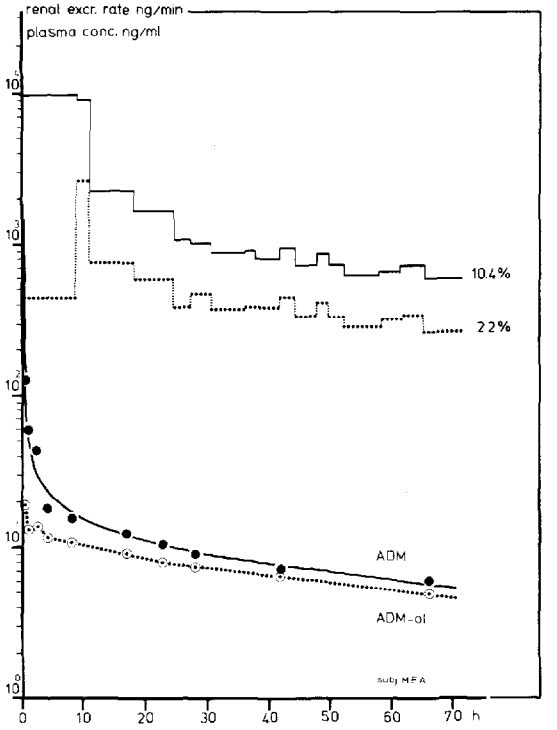

Fig. 4. The plasma concentration—time profiles of ADM (•) and of ADM-ol (○), measured over a period of 66 h, from a patient given 1.22 mg/kg by i.v. bolus injection. The renal excretion rate—time profiles of ADM (——) and of ADM-ol (• • •) are also shown.

A linear relationship between the renal excretion rates (ng/min) and the plasma concentrations (ng/ml) 10 h after administration were found for all four compounds. Two typical examples for ADM and ADM-ol are given in Fig. 6. The average renal clearance values (ml/min) as well as some pharmacokinetic parameters are listed in Table II.

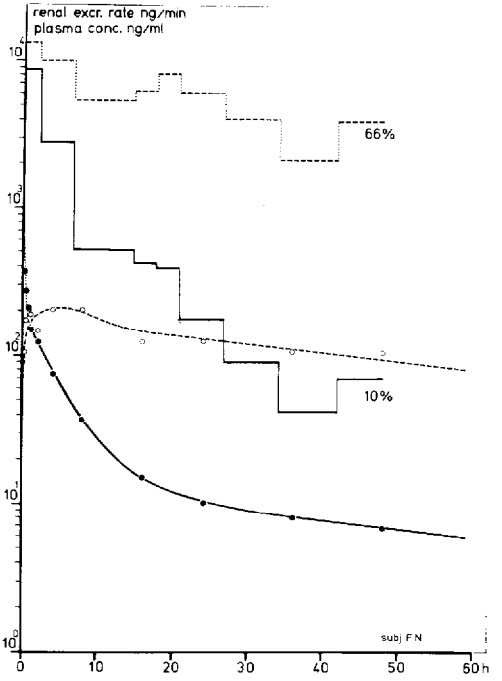

Fig. 5. The plasma concentration—time profiles of CAM (•) and of CAM-ol (○), measured over a period of 50 h, from a patient given 0.36 mg/kg by i.v. bolus injection. The renal excretion rate—time profiles of CAM (——) and of CAM-ol (• • •) are also shown.

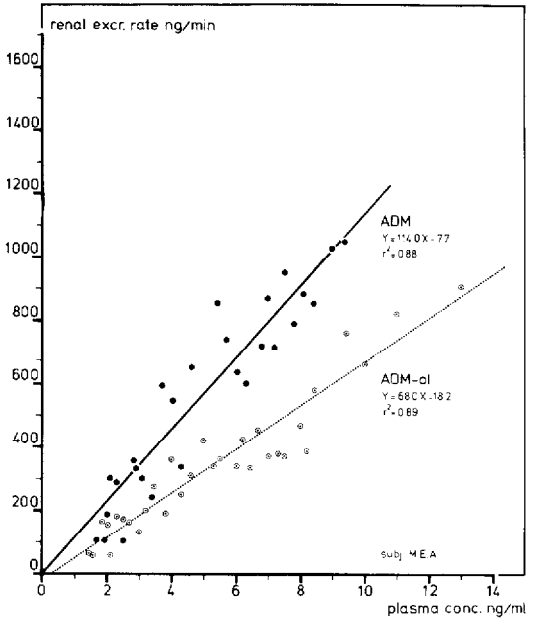

Fig. 6. The renal excretion rates versus plasma concentrations of ADM and of ADM-ol of a patient. The renal clearance of ADM was 114 ml/min, that of ADM-ol 68 ml/min.

TABLE II

PHARMACOKINETIC PARAMETERS OF ADRIAMYCIN AND CARMINOMYCIN IN MAN AFTER i.v. BOLUS ADMINISTRATION

Abbreviations: $t_{½}$ = half life time of terminal phase; V_f = apparent distribution volume; Cl_p = total body clearance; AUC_p = area under the curve of parent compound; AUC_m = area under the curve of hydroxy metabolite; Cl_{rp} = renal clearance of parent compound; Cl_{rm} = renal clearance of hydroxy metabolite; $rec._p$ = recovery of parent compound from urine; $rec._m$ = recovery of hydroxy metabolite from urine.

Drug	$t_{½}$ (h)	V_f (l)	Cl_p (ml/min)	AUC_p^* (ug.h/l)	AUC_m^* (ug.h/l)	Cl_{rp} (ml/min)	Cl_{rm} (ml/min)	$rec._p^{**}$ (%)	$rec._m^{**}$ (%)
ADM	54	1380	640	2760	1100	114	68	10	2.2
CAM	47	500	230	1800	14100	23	46	10	66

*The AUC values are extrapolated to $t = \infty$.
**The recoveries are calculated from the measured period of time.

DISCUSSION

From Fig. 4, it can be seen that the plasma concentrations of ADM and ADM-ol decline below 10 ng/ml within 10 h after i.v. bolus administration. Thereafter the plasma concentrations of ADM and ADM-ol decline with a half-life time ($t_{½}$) of 50 h. With the described method, it is possible to determine plasma concentrations of ADM after administration of conventional dosages for several days. The same holds true for CAM (Fig. 5). As the concentration of the anthracyclines in urine are higher than in plasma, there will be no problems using this method in determining urine concentrations during the week following administration, since the detection limit is 0.5 ng/ml (Eksborg et al. [10], 2 ng/ml).

In a recent article by Eksborg et al. [18], a decreased ADM plasma concentration was measured after ten thawings and refreezings. The concentration declined from 1000 to 50 ng/ml. After a storage time of six months at −20°C, the concentration had declined from 250 to 150 ng/ml.

In our experiment, however, no decrease in plasma concentration could be observed after a storage time fourteen days and after seven thawings and refreezings.

The structural difference between ADM and CAM results in an increased plasma concentration of the hydroxy metabolite of CAM, CAM-ol. At 20 h after administration, the ratio of the plasma concentrations ADM/ADM-ol was 1.11, that of CAM/CAM-ol 0.073. The renal clearances of ADM-ol and CAM-ol are in the same range: ADM-ol, 68 ml/min; CAM-ol, 46 ml/min. The high plasma concentration of CAM-ol explains the high recovery in urine (66%). Whether an increased affinity for the reducing enzymes of CAM or a lower volume of distribution of CAM-ol are the cause for its higher plasma concentration still needs to be investigated.

No glucuronides of the parent compounds or of the hydroxy metabolites have been found in plasma or urine. In case of ADM, excretion occurs mainly along the biliary route. This was confirmed in a dog experiment, where ± 50%

appeared to be excreted via this way measured over a period of three days (unpublished results).

The described method has been applied to study pharmacokinetics of ADM in man [19] and to study structure related pharmacokinetics of the anthracyclines in dogs [20].

REFERENCES

1 M. Dubost, P. Ganter, R. Maral, L. Ninet, S. Pinnert, J. Preud'Homme and G.H. Werner, C.R. Acad. Sci., 257 (1963) 1813.
2 F. Arcamone, Doxorubicin Anticancer Antibiotics (Medicinal Chemistry, Vol. 17), Academic Press, New York, 1981, p. 145.
3 N.R. Bachur, A.L. Moore, J.B. Bernstein and A. Liu, Cancer Chemother. Rep., 54 (1970) 89.
4 D.S. Alberts, N.R. Bachur and J.L. Holtzman, Clin. Pharmacol. Ther., 12 (1971) 96.
5 N.R. Bachur, R.C. Hildebrand and R.S. Jaencke, J. Pharmacol. Exp. Ther., 191 (1974) 331.
6 R.S. Benjamin, C.E. Riggs and N.R. Bachur, Clin. Pharmacol. Ther., 14 (1973) 592.
7 R. Hulhoven and J.P. Desager, J. Chromatogr., 125 (1976) 369.
8 M. Israel, W.J. Pegg, P.M. Wilkinson and M.B. Garmick, J. Liquid Chromatogr., 1 (1978) 795.
9 R.N. Pierce and P.I. Yetlow, J. Chromatogr., 164 (1979) 471.
10 S. Eksborg, H. Ehrsson and I. Andersson, J. Chromatogr., 164 (1979) 479.
11 R. Baurain, A. Zenebergh and A. Trouet, J. Chromatogr., 157 (1978) 331.
12 E. Moro, V. Bellotti, M.G. Jannuzzo, S. Stegnjaich and G. Valzelli, J. Chromatogr., 274 (1983) 281.
13 M.A. Van Lancker, H.J.C.F. Nelis and A.P. De Leenheer, J. Chromatogr., 254 (1983) 45.
14 H. van Vunakis, J.J. Langone, L.J. Riceberg and L. Levine, Cancer Res., 34 (1974) 2546.
15 M.J.M. Oosterbaan, M.J.M. Dirks, T.B. Vree and E. van der Kleijn, in P. Periti and G.G. Grassi (Editors), Proceedings of the 12th International Congress of Chemotherapy, Florence, Italy, American Society for Microbiology, Washington, DC, 1981, p. 1445.
16 J. Lankelma, E. van der Kleijn and M.J.T. Jansen, J. Chromatogr., 182 (1980) 35.
17 J.G. Wagner, Fundamentals of Clinical Pharmacokinetics, Drug Intelligence Publ., Hamilton, IL, 1975.
18 S. Eksborg, H. Ehrsson, I. Wallin and A. Lindfors, Acta Pharm. Suecica, 18 (1981) 215.
19 M.J.M. Oosterbaan, M.J.M. Dirks, T.B. Vree and E. van der Kleijn, submitted for publication.
20 M.J.M. Oosterbaan, M.J.M. Dirks, T.B. Vree and E. van der Kleijn, Pharm. Res., 1 (1984) 33.