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Simultaneous determination of etretinate, acitretin and their metabolites in perfusate, perfusate plasma, bile or hepatic tissue with reversed-phase high-performance liquid chromatography

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Abstract

Etretinate is a synthetic aromatic retinoid used in the treatment of psoriasis and other disorders affecting the skin. Acitretin is the primary active metabolite of etretinate. The in situ perfused rat liver model was used to study the first-pass hepatic metabolism of etretinate and acitretin and a reliable method of quantifying etretinate and its metabolites was needed. Previously published assays allow for the simultaneous quantitation of etretinate and acitretin in blood or plasma. This paper describes an accurate and reliable reversed-phase HPLC method for the determination of etretinate, acitretin and their metabolites in whole perfusate, plasma, bile and hepatic tissue.

1. Introduction

Etretinate (ET, Ro 10-9359) is a synthetic aromatic derivative of vitamin A that is used for the treatment of generalized severe psoriasis and other hyperkeratotic disorders affecting the skin [1]. Acitretin (ETA, Ro 10-1670) is the primary and active metabolite of ET and has also been proven effective in the treatment of psoriasis and other skin diseases [2]. The absolute oral bioavailabilities of ET and ETA in humans are approximately 40 and 60% respectively [3] and studies in the dog have indicated that first-pass hepatic metabolism may play an important role in the overall loss of oral bioavailability of ET [4]. The in situ perfused rat liver was used as an animal model to further characterize the first-pass hepatic elimination and hepatic uptake of ET and ETA. Although many analytical assays for the quantitation of ET and ETA in blood, plasma, or urine have been developed, none of these published methods also allow for the quantitation of ET and its metabolites in hepatic tissue [5–14]. The objective of this study was to develop a reliable and sensitive analytical method for the quantitation of ET, ETA, the 13-*cis* isomer of acitretin (c-ETA) and the glucuronide conjugates of ETA and c-ETA in whole perfusate, perfusate plasma, bile and hepatic tissue. This was carried out by modifying the sample

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preparation and HPLC methods developed by Thongnopnua and Zimmerman [14].

2. Experimental

2.1. Analytical standards and chemicals

ET, ETA and c-ETA were provided by Hoffmann-La Roche (Nutley, NJ, USA). The internal standard, retinyl acetate, was obtained from Sigma (St. Louis, MO, USA). All retinoids were stored in amber containers at −5°C under a vacuum. Handling of these compounds and biological samples was carried out in a room with yellow lighting to prevent photoisomerization.

HPLC grade acetonitrile and methanol were obtained from EM Scientific (Gibbstown, NJ, USA). Dipotassium hydrogen phosphate trihydrate was obtained from Mallinckrodt (Paris, KY, USA). HPLC grade glacial acetic acid, HPLC grade ammonium acetate and 1-butanol were obtained from Fisher Scientific (Fairlawn, NJ, USA). Butylated hydroxytoluene (BHT) and β-glucuronidase solution (from *Helix pomatia*; 100 000 units/ml) were obtained from Sigma.

2.2. Preparation of calibration solutions

Stock solutions of ET, ETA and c-ETA (250 μg/ml) in methanol–acetonitrile (1:1, v/v) containing BHT (50 μg/ml) as an antioxidant were used for preparing the calibration solutions. A series of calibration solutions containing ET and ETA were prepared by serial dilution of the stock solutions to concentrations of 0.8, 2.0, 4.0, 8.0, 10.0, 20.0, 50.0 and 100.0 μg/ml. These calibration solutions were used in the analysis of whole perfusate and plasma samples. For the analysis of bile samples a similar set of calibration solutions containing ETA and c-ETA was prepared. ET was not included in this set of solutions as unchanged ET was not found in the bile. A third set of calibration solutions containing ET, ETA and c-ETA was prepared for the analysis of tissue homogenate samples as all three were found in the liver tissue. The concentrations of ET and ETA in this set of calibration solutions were 8.0, 10.0, 20.0, 50.0, 100.0 and 200.0 μg/ml. The concentrations of c-ETA in these solutions were one-fourth that of ET and ETA reflecting the lower concentrations of c-ETA in the tissue samples. A working solution of retinyl acetate (internal standard, I.S.) was prepared by diluting 10 ml of a stock solution (retinyl acetate 376 μg/ml) to a final concentration of 37.6 μg/ml. The calibration and working solutions were prepared every 1 to 2 months. All solutions were stored at −5°C in foil wrapped flasks.

2.3. Perfusion experiment and sampling

ET or ETA was perfused in a red blood cell based perfusate through the in situ rat liver preparation at concentrations of 2.0, 5.0 or 10.0 μg/ml to study the first-pass metabolism and hepatic distribution of both compounds. Perfusate and bile samples were collected throughout the 120-min studies. A single steady-state hepatic tissue sample was obtained at the conclusion of the experiment. The red blood cell based perfusate used was similar to that described by Pang [15].

2.4. Preextraction sample work-up

All perfusate and plasma samples were frozen until extraction, at which time they were thawed at room temperature and vortex-mixed for 10 s. They were then refrozen in a mixture of dry ice and acetone and again thawed.

Bile samples were diluted with an equal volume of 0.2 *M* sodium acetate (pH 5) at the time of collection. A hydrolysis procedure allowed for the indirect quantitation of the glucuronide conjugates of ETA and c-ETA in the bile. The sample bile was split into two portions, one of which was hydrolyzed and the other which served as a control. The hydrolyzed sample was prepared by adding 100 μl of diluted sample and 8 μl of β-glucuronidase solution (100 000 units/ml) to a 1.5-ml microcentrifuge tube. The sample was then incubated at 37°C in a waterbath for 5 h. The control sample consisted of 100 μl of

diluted sample which was kept at room temperature during the incubation of the hydrolyzed sample. All samples were then frozen until the time of extraction.

The hepatic tissue samples were prepared as follows: a 1-g piece of tissue was weighed, minced and placed in a glass homogenization tube. Then 4 ml of ice-cold Krebs–Henseleit bicarbonate buffer (pH 7.4) was added and the mixture was homogenized by moving the homogenizer, a hand-held electric drill equipped with a PTFE-coated homogenization bit, through the mixture several times. The entire process was carried out on ice to insure that minimal metabolism took place in the tissue sample. The tissue homogenate for the standard curve was prepared in a similar manner using a piece of blank liver tissue.

2.5. Extraction procedure

The calibration curves were prepared by adding 100 μl of blank perfusate, plasma, diluted bile or tissue homogenate to 1.5-ml microcentrifuge tubes. Each tube was then spiked with 20 μl of the appropriate calibration solution. Similarly, 20 μl of acetonitrile was added to tubes containing 100 μl of perfusate, bile or tissue sample. Then, 350 μl of freshly prepared acetonitrile–1-butanol (1:1, v/v) were added to each tube followed by the addition of 20 μl of working I.S. solution. The tubes were vortex-mixed for 1 min followed by the addition of 300 μl of aqueous dipotassium hydrogen phosphate solution (1 g/ml). The samples were then vortex-mixed for 30 s and centrifuged for 3 min at 13 600 g (Microcentrifuge Model 235B, Fisher Scientific). The organic layer (200 μl) was then transferred to vials for injection onto the HPLC system and the aqueous layer was discarded. The containers for HPLC consisted of a polyethylene insert in an amberized glass autosampler vial capped with a open screw cap with a PTFE seal (Sunbrokers, Wilmington, NC, USA).

2.6. Chromatographic equipment and conditions

The HPLC analysis was conducted using a Model 6000A chromatographic pump, a WISP autoinjector (both from Waters Associates, Milford, MA, USA), a Model SPD-6A UV spectrophotometric detector and a Model C-R6A Chromatopac integrator (both from Shimadzu, Kyoto, Japan). The HPLC column used was a Supelcosil LC-18 column (15 cm × 4.6 mm I.D., 5 μm particle size) preceded by a 1-cm Supelcosil LC-18 guard column (Supelco, Bellefonte, PA, USA). The mobile phase consisted of approximately 79% acetonitrile in acetate buffer, and was prepared by adding 0.8 g of ammonium acetate and 10 ml of glacial acetic acid to 800 ml of acetonitrile and 200 ml distilled water. The flow-rate of the mobile phase was 1.5 ml/min and the column was heated to 50°C with a block column heater (Timberline Instruments, Boulder, CO, USA). The UV detection was carried out at a wavelength of 350 nm at 0.01 AUFS. The chart speed was 0.5 cm/min.

The bile and tissue samples were analyzed with a reversed-phase gradient-elution HPLC assay that allowed the separation of c-ETA, ETA and the glucuronide metabolites. The chromatographic equipment was the same as that used for the isocratic assay except that a second Model 6000A pump and a Model 660 solvent programmer were added (Waters Associates). A Supelcosil LC-18 column (25 cm × 4.6 mm I.D., 5 μm particle size) and a 1-cm LC-18 guard column (Supelco) were used for this analysis.

The mobile phases used for the gradient-elution method were 20% acetonitrile in acetate buffer (mobile phase A) and 100% acetonitrile (mobile phase B). To separate only c-ETA and ETA, the initial conditions and final conditions were 74% acetonitrile (69% B) and 88% acetonitrile (90% B) respectively. A step function gradient (No. 11) was used where the initial conditions were held for 8 min before switching to the final conditions for the remainder of the individual run. The column was allowed to re-equilibrate for 6 min between runs. To facilitate the separation of the glucuronide peak from the solvent front, the initial and final conditions were changed to 48% acetonitrile (35% B) and 75% acetonitrile (69% B) respectively. The initial conditions were held for 10 min before switching

to the final conditions. A 6-min equilibration delay was again employed. The flow-rate, column temperature, chart speed and detector settings were the same as for the isocratic HPLC analysis.

2.7. Assay validation

Calibration curves consisting of eight different concentrations for perfusate and bile analysis and six different concentrations for tissue analysis were generated by linear regression of the peak-area ratios (drug/internal standard) on the concentrations of the drugs. The natural logs of the peak-area ratios and concentrations were used in this analysis to insure the linearity of the plot. The unknown drug concentrations in biological samples were determined from the equations generated by this least-squares regression analysis.

The with-in and between-day precision as well as the accuracy of the assay were determined by standard means. The within-day precision was determined from a triplicate standard curve on a given day. The between-day recovery was determined from single standard curves run on different days throughout the months of the liver perfusion studies. The recovery of each compound at each concentration was determined in a triplicate standard curve. The peak areas of the compounds in the extracted samples were compared with those of the calibration solution injected directly onto the HPLC after dilution. The percent recovery of each compound was calculated as: recovery (%) = [(peak area of extracted sample/injected volume)/(peak area of non-extracted sample/injected volume)] · 100.

The precision and accuracy of the assay were determined in whole perfusate, plasma, bile and tissue homogenate and the recovery of each compound during extraction was determined in whole perfusate, bile and tissue homogenate.

3. Results and discussion

The isocratic HPLC method allowed the separation of ETA and ET with a run time of

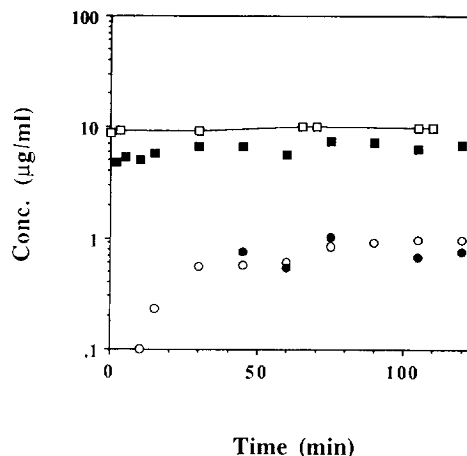

Fig. 1. Whole perfusate concentration–time profile after single-pass liver perfusion with etretinate at a concentration of 10 μg/ml. The reservoir concentration of ET (□) and outflow perfusate concentrations of ET (■), ETA (○) and unknown metabolite (●) are shown.

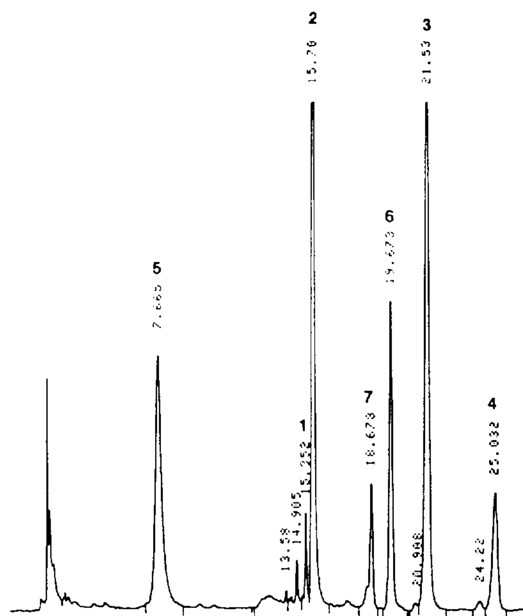

Fig. 2. HPLC profile of hepatic tissue sample. Peaks: 1 = *cis*-acitretin (8.00 μg/ml); 2 = acitretin (91.82 μg/ml); 3 = etretinate (67.48 μg/ml); 4 = retinyl acetate (internal standard); 5 = glucuronides (c-ETA and ETA); 6 = unknown metabolite; 7 = endogenous compound.

Table 1
Analytical precision and accuracy for etretinate and acitretin analysis in whole perfusate

Theoretical concentration (μg/ml)	Within-day (n = 3)		Between-day			Accuracy (%)
	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	n	C.V. (%)	
Etretinate						
0.160	0.176 ± 0.019	10.8	0.164 ± 0.200	6	11.9	1.0
0.400	0.390 ± 0.012	3.1	0.409 ± 0.016	8	3.9	0.7
0.800	0.784 ± 0.043	5.5	0.851 ± 0.096	6	11.3	2.5
1.600	1.523 ± 0.055	3.6	1.602 ± 0.126	8	7.8	1.0
2.000	1.883 ± 0.071	3.8	2.071 ± 0.223	10	10.7	0.9
4.000	3.993 ± 0.152	3.8	4.023 ± 0.295	10	7.3	0.5
10.000	9.995 ± 0.710	7.1	10.073 ± 1.110	10	11.0	0.5
20.000	21.536 ± 2.216	10.3	19.729 ± 1.341	10	6.8	0.9
Acitretin						
0.160	0.153 ± 0.013	8.5	0.165 ± 0.025	6	14.9	2.2
0.400	0.434 ± 0.062	8.3	0.412 ± 0.046	8	11.1	0.2
0.800	0.769 ± 0.000	0.0	0.792 ± 0.066	6	8.3	2.6
1.600	1.583 ± 0.048	3.0	1.522 ± 0.203	8	13.3	0.9
2.000	2.048 ± 0.053	2.6	2.160 ± 0.231	10	10.7	1.2
4.000	4.039 ± 0.094	2.3	4.303 ± 0.784	10	18.2	1.6
10.000	10.159 ± 0.791	7.8	10.358 ± 0.657	10	6.3	0.8
20.000	19.525 ± 1.942	9.9	20.202 ± 1.790	10	8.9	0.7

Table 2
Analytical precision and accuracy for acitretin and *cis*-acitretin analysis in bile

Theoretical concentration (μg/ml)	Within-day (n = 3)		Between-day (n = 4)		Accuracy (%)
	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	
Acitretin					
0.160	0.158 ± 0.005	2.9	0.157 ± 0.005	3.5	1.6
0.400	0.406 ± 0.109	4.7	0.407 ± 0.013	3.3	3.9
0.800	0.800 ± 0.270	3.3	0.772 ± 0.057	7.4	4.2
1.600	1.607 ± 0.072	4.5	1.631 ± 0.034	2.1	2.6
2.000	2.023 ± 0.064	3.2	2.038 ± 0.042	2.0	1.5
4.000	3.890 ± 0.201	5.2	4.014 ± 0.191	4.7	0.4
10.000	10.260 ± 0.115	1.1	9.852 ± 0.491	5.0	0.6
20.000	19.744 ± 0.324	1.6	–	–	1.3
cis-Acitretin					
0.160	0.161 ± 0.003	2.2	0.155 ± 0.009	5.7	1.7
0.400	0.403 ± 0.008	1.9	0.411 ± 0.017	4.2	5.0
0.800	0.785 ± 0.011	1.4	0.745 ± 0.044	5.9	4.3
1.600	1.590 ± 0.035	2.2	1.632 ± 0.078	4.8	0.5
2.000	1.889 ± 0.264	14.0	2.063 ± 0.090	4.4	0.4
4.000	3.916 ± 0.215	5.5	4.019 ± 0.182	4.5	0.2
10.000	10.377 ± 0.053	0.5	9.858 ± 0.557	5.7	0.2
20.000	19.710 ± 0.192	1.0	–	–	1.5

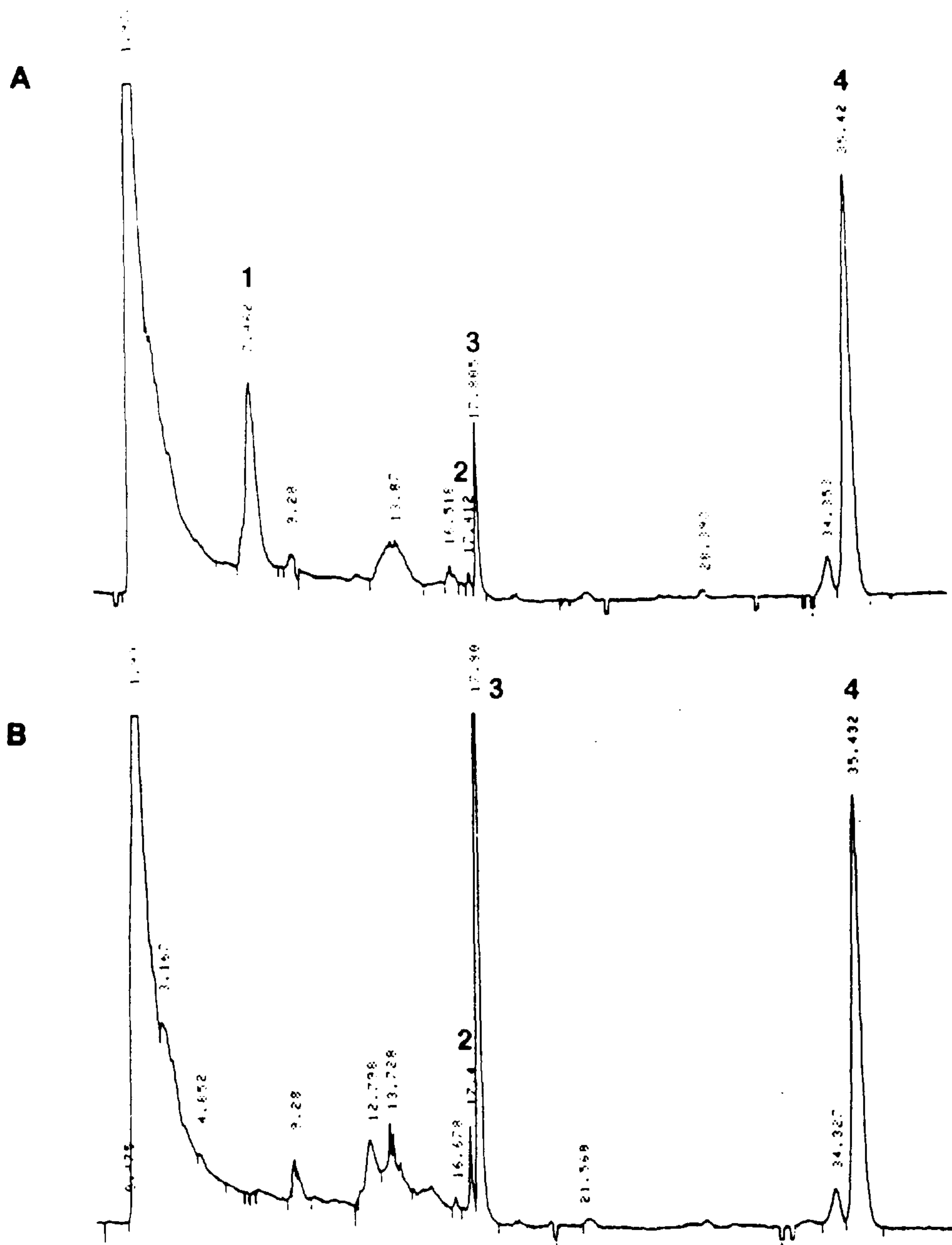

Fig. 3. HPLC profile of (A) non-hydrolyzed bile sample and (B) hydrolyzed bile sample. Peaks: 1 = glucuronides (c-ETA and ETA); 2 = *cis*-acitretin (0.248 μg/ml in B); 3 = acitretin (0.802 μg/ml in A, 3.968 μg/ml in B); 4 = retinyl acetate (internal standard).

approximately 12 min. The retention times of ETA, an unknown metabolite, ET and I.S. were 2.8, 5.3, 6.5 and 8.7 min, respectively. No interfering peaks were noted. A typical perfusate concentration–time profile for a liver perfusion experiment with ET is shown in Fig. 1.

The gradient-elution technique with initial and final conditions of 74 and 88% acetonitrile respectively, was efficient in separating the *cis* and *trans* isomers of ETA although the run time was increased to 16 min. The respective retention times of c-ETA, ETA, the unknown metabolite, ET and I.S. under these conditions were 5.5, 6.0, 12.1, 13.0 and 14.3 min, respectively (data not shown). When the gradient parameters were altered to allow the separation and direct quantitation of the glucuronide peak the run time was further increased to 27 min. The retention times of the glucuronide metabolites (of c-ETA and ETA), c-ETA, ETA, the unknown metabolite, ET and I.S. using this extended gradient technique were 7.6, 15.3, 15.7, 19.7, 21.5 and 25.1 min, respectively. It was not possible to separate the glucuronide peak into the *cis* and *trans* components using this method.

Fig. 2 shows a chromatogram of a typical tissue homogenate sample. In addition to the drug peaks in these samples an endogenous peak was seen with a retention time between those of ETA and ET (18.7 min). This peak was also seen in samples of blank hepatic tissue homogenate. Typical chromatograms of non-hydrolyzed (control) and hydrolyzed bile samples are shown in Fig. 3. There were no interfering peaks with either of the two gradient-elution methods. The differences in the retention times between Fig. 2 and Fig. 3 are due to slight changes in the analytical conditions over time as the two chromatograms were generated several months apart.

The summaries of the analytical precision and accuracy in the calibration curves in whole perfusate, bile and tissue homogenate appear in Tables 1–3, respectively. Similar results were observed for perfusate plasma (data not shown). The coefficients of variation (%C.V.) of the total variability for ET, ETA and c-ETA varied randomly between 1 and 20% in all media although the C.V.s were for the most part be-

Table 3
Analytical within-day precision and accuracy for etretinate, acitretin and *cis*-acitretin analysis in liver tissue homogenate

Theoretical concentration (μg/ml)	Mean concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (%)
Etretinate (n = 3)			
1.60	1.590 ± 0.062	3.9	1.1
2.00	1.987 ± 0.049	2.5	0.7
4.00	4.093 ± 0.080	2.0	1.7
10.00	9.733 ± 0.218	2.2	1.7
20.00	20.776 ± 0.566	2.7	2.1
40.00	39.217 ± 1.175	3.0	0.9
Acitretin (n = 4)			
1.60	1.626 ± 0.073	4.5	1.4
2.00	2.085 ± 0.082	3.9	1.2
4.00	4.044 ± 0.056	1.4	6.3
10.00	8.825 ± 0.157	1.8	2.3
20.00	21.375 ± 0.745	3.5	0.4
40.00	40.624 ± 1.346	3.3	0.5
cis-Acitretin (n = 3)			
0.40	0.448 ± 0.024	5.4	4.3
0.50	0.490 ± 0.058	11.8	1.6
1.00	0.944 ± 0.035	3.7	0.1
2.50	2.186 ± 0.152	7.0	5.7
5.00	5.252 ± 0.266	5.1	1.5
10.00	10.609 ± 0.718	6.8	5.5

tween 5 and 10% throughout the concentration range. The C.V.s appeared to remain constant throughout the concentration range of the standard curves in all biological matrices. The assay was accurate within 3% of the theoretical concentration at all levels of concentration in all matrices with a few exceptions (range: <0.1% to 6.3%).

The recoveries of ET, ETA and c-ETA from whole perfusate, bile and tissue homogenate are shown in Tables 4 and 5. The overall percent recoveries (mean ± standard deviation) of ET and ETA in whole perfusate were 86.5 ± 7.5 and 86.1 ± 7.3%, respectively. In the extraction from bile the percent recoveries (mean ± S.D.) of ETA and c-ETA were 90.3 ± 8.0 and 87.0 ± 6.4%, respectively. The highest recoveries of drug were from the tissue homogenate samples with percent recoveries (mean ± S.D.) for ET, ETA and c-ETA of 99.4 ± 7.7, 96.5 ± 10.5 and

Table 4
Recovery of etretinate, acitretin and cis-acitretin from whole perfusate or bile

Concentration (μg/ml)	Perfusate		Bile	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Etretinate				
0.160	84.9 ± 4.0[a]	4.7		
0.400	88.0 ± 2.7	3.1		
0.800	81.7 ± 3.6	4.4		
1.600	90.5 ± 7.6	8.4		
2.000	96.3 ± 7.2	7.5		
4.000	85.8 ± 5.6	6.5		
10.000	85.5 ± 10.5	12.3		
20.000	77.5 ± 5.7	7.3		
Overall[b]	86.5 ± 7.5	8.7		
Acitretin				
0.160	88.6 ± 5.6	6.3	101.6 ± 4.1	4.0
0.400	88.1 ± 1.2	1.3	93.4 ± 1.3	1.4
0.800	78.2 ± 3.2	4.1	88.1 ± 4.0	4.5
1.600	89.4 ± 6.4	7.1	91.7 ± 11.5	12.5
2.000	93.9 ± 4.7	5.0	86.0 ± 2.5	2.9
4.000	85.4 ± 6.2	7.3	87.8 ± 1.0	1.1
10.000	87.1 ± 10.4	11.9	95.8 ± 4.2	4.4
20.000	78.0 ± 6.5	8.3	82.0 ± 10.5	12.9
Overall[b]	86.1 ± 7.3	8.5	90.3 ± 8.0	8.9
cis-Acitretin				
0.160			85.4 ± 5.4	6.3
0.400			88.3 ± 1.5	1.7
0.800			90.2 ± 3.1	3.5
1.600			90.1 ± 10.8	11.9
2.000			85.9 ± 2.5	2.9
4.000			84.7 ± 0.2	0.2
10.000			93.3 ± 3.2	3.4
20.000			78.2 ± 9.1	11.6
Overall[b]			87.0 ± 6.4	7.4

[a] Mean ± S.D., $n = 3$ at each level.
[b] The overall mean, S.D. and %C.V. were calculated from the individual data.

Table 5
Recovery of etretinate, acitretin and *cis*-acitretin from hepatic tissue homogenate

Concentration (μg/ml)	Recovery (mean ± S.D.[a]) (%)	C.V. (%)
Etretinate		
1.600	100.3 ± 3.1	3.1
2.000	108.2 ± 12.5	11.6
4.000	92.4 ± 1.6	1.7
10.000	99.7 ± 4.4	4.5
20.000	99.2 ± 2.7	2.7
40.000	96.3 ± 9.9	10.3
Overall[b]	99.4 ± 7.7	7.7
Acitretin		
1.600	103.0 ± 2.7	2.7
2.000	110.2 ± 16.6	15.1
4.000	88.2 ± 2.1	2.3
10.000	95.3 ± 4.8	5.0
20.000	93.0 ± 1.8	2.0
40.000	89.4 ± 9.4	10.5
Overall[b]	96.5 ± 10.5	10.9
cis-Acitretin		
0.400	86.5	–
0.500	106.3 ± 15.4	14.5
1.000	107.2 ± 8.4	7.8
2.500	99.9 ± 7.5	7.5
5.000	92.1 ± 0.6	0.6
10.000	87.3 ± 5.3	6.1
Overall[b]	97.8 ± 11.1	11.3

[a] $n = 3$ at each level, except 13-c-ETA 0.4 μg/ml ($n = 1$).
[b] The overall mean, S.D. and %C.V. were calculated from the individual data.

97.8 ± 11.1%, respectively. The recovery of ET, ETA and c-ETA from whole perfusate, bile and tissue homogenate was independent of drug concentration ($p > 0.05$), except for the recovery of ETA from tissue which had a p-value of 0.044. However, the Scheffé test statistic was not significant between any two groups at the 95% level.

Although other reversed- and normal-phase HPLC methods for the separation of ET and ETA have been published previously, the present method allows for the precise and accurate quantitation of ET and its metabolites in bile and hepatic tissue samples with only minor modifications to the method used for the analysis of whole perfusate and plasma samples. The gradient-elution method allowed the separation of ETA and c-ETA and their glucuronide conjugates with a total run time per sample of less than 30 min.

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References

[1] A.S. Boyd, *Am. J. Med.*, 86 (1989) 568.
[2] T.J. O'Brien, *Int. J. Dermatol.*, 29 (1990) 270.
[3] F.G. Larsen, F. Nielsen-Kudsk, P. Jakobsen, K. Weismann and K. Kragballe, *Clin. Pharmacokinet.*, 23 (1992) 42.
[4] P. Thongnopnua, J.W. Massarella and C.L. Zimmerman, *Drug Metab. Disp.*, 17 (1989) 473.
[5] R. Hänni, D. Hervouet and A. Busslinger, *J. Chromatogr.*, 162 (1979) 615.
[6] U. Paravicini and A. Busslinger, *J. Chromatogr.*, 276 (1983) 359.
[7] A.P. De Leenheer, W.E. Lambert, J.P. De Bersaques and A.H. Kint, *J. Chromatogr.*, 500 (1990) 637.
[8] E. Meyer, W.E. Lambert and A.P. De Leenheer, *J. Chromatogr.*, 570 (1991) 149.
[9] G. Palmskog, *J. Chromatogr.*, 221 (1980) 345.
[10] J. Besner, S. Meloche, R. Leclaire, P. Band and S. Mailhot, *J. Chromatogr.*, 231 (1982) 467.
[11] C.J.L. Buggé, L.C. Rodriguez and F.M. Vane, *J. Pharm. Biomed. Anal.*, 3 (1985) 269.
[12] P. Jakobsen, F.G. Larsen and C.G. Larsen, *J. Chromatogr.*, 415 (1987) 413.
[13] N.R. Al-Mallah, H. Bun, P. Coassolo, C. Aubert and J.P. Cano, *J. Chromatogr.*, 421 (1987) 177.
[14] P. Thongnopnua and C.L. Zimmerman, *J. Chromatogr.*, 433 (1988) 345.
[15] K.S. Pang, in J.R. Mitchell and M.G. Horning (Editors), *Drug Metabolism and Drug Toxicity*, Raven Press, New York, 1984, p. 331.