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Quantification of clarithromycin, its 14-hydroxy and decladinose metabolites in rat plasma, gastric juice and gastric tissue using high-performance liquid chromatography with electrochemical detection

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Abstract

A rapid, selective and sensitive HPLC assay has been developed for the simultaneous analysis of clarithromycin, its 14-hydroxy-clarithromycin metabolite, and its decladinose acid degradation product, in small volumes of rat gastric juice aspirate, plasma and gastric tissue. Sample were extracted with *n*-hexane/2-butanol (4:1) and the internal standard was roxithromycin. A Kromasil ODS 5 μm (75×4.6 mm I.D.) column was used with a mobile phase consisting of acetonitrile/aqueous phosphate buffer (pH 7, 0.086 *M*) (45:55 v/v). The column temperature was 30 °C and coulometric detection was used at 850 mV using a screen voltage of 600 mV. The analysis time was less than 8 min. The limits of quantitation for clarithromycin, 14-OH clarithromycin and decladinose clarithromycin were 0.15 μg ml^{-1} or lower in plasma (0.05 ml); 0.16 μg ml^{-1} or lower in gastric juice (0.2 ml); and 0.51 μg g^{-1} or lower for gastric tissue (0.25 g). The method was linear up to at least 20.3, 15.4 and 12.5 μg ml^{-1} for clarithromycin, 14-OH-clarithromycin and decladinose, respectively, in gastric juice aspirate and plasma and up to 40.6, 30.9 and 25.0 μg g^{-1} in gastric tissue. The assay was applied to the measurement of clarithromycin, 14-OH-clarithromycin and, for the first time, decladinose clarithromycin in pharmacokinetic studies of gastric transfer of clarithromycin in individual rats.

Keywords: Clarithromycin; 14-Hydroxy-clarithromycin; Decladinose clarithromycin; Antibiotics

1. Introduction

Clarithromycin [6-*O*-methylerythromycin A, Fig. 1] is an acid-stable, semisynthetic macrolide antibiotic drug that displays good antimicrobial activity against a wide range of Gram-positive and Gram-negative organisms [1]. It is primarily metabolised to its biologically active 14-hydroxy-6-*O*-methylerythromycin metabolite (14-OH-clarithromycin, Fig. 1) in animals as well as in man [2]. It can be inactivated by the hydrolytic removal of the cladinose sugar moiety, which takes place under acidic conditions yielding the decladinose acid degradation product [5-*O*-desosaminyl-6-*O*-methylerythronolide A] [3–5]. No analytical HPLC method has yet been reported for the routine analysis of this

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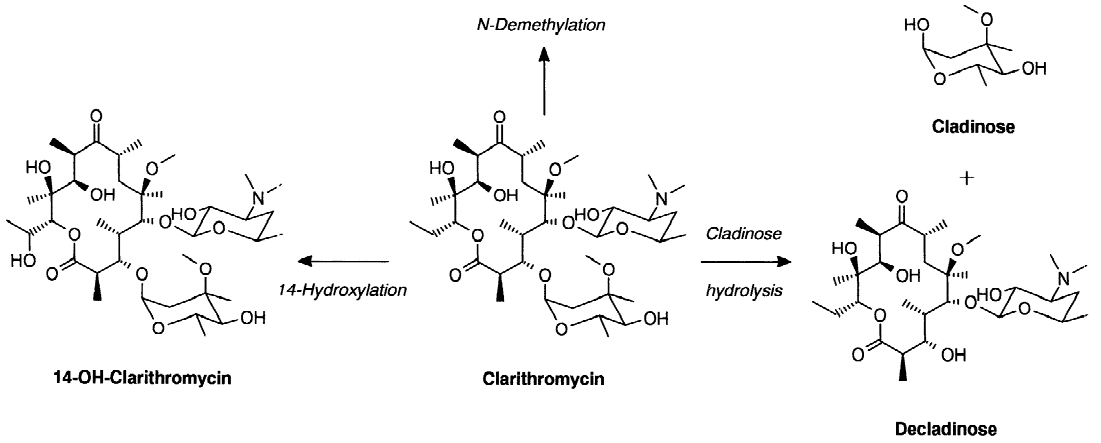

Fig. 1. Metabolism of clarithromycin to form 14-OH-clarithromycin and acid degradation to form decladinose clarithromycin and cladinose (after Ferrero et al. [2]).

decladinose acid degradation product despite the likely occurrence of this compound in the highly acidic environment of the stomach following oral administration of clarithromycin.

Clarithromycin is now widely used in a standard eradication treatment of gastric *Helicobacter pylori* infections, where it is combined with an acid-suppressing agent and a second antibiotic [6]. In order to eradicate *H. pylori* effectively, clarithromycin has to reach the minimum inhibitory concentration (MIC) of 0.75 μg ml^{-1} in an acidic environment (pH 5.5) [7]. The determination of clarithromycin levels in gastric juice and the extent to which it degrades under acidic conditions, is therefore crucial to ensure an effective pharmaco-therapeutic regimen against gastric *H. pylori* infections [8]. The mechanism of clarithromycin transfer to the stomach from the systemic circulation after parenteral administration and the extent to which acid degradation occurs during transfer are important factors which influence effective antibacterial therapy [9,10].

Methods reported previously for the detection of clarithromycin have mainly used sample preparation procedures involving liquid–liquid extraction [2,11–13]or solid-phase extraction [14] prior to HPLC analysis. Electrochemical [15] or UV [16] or electrospray mass spectrometry [17] detection have been used in combination with simple buffered methanol or acetonitrile mobile phases, and ion-pair HPLC assay for assessment of clarithromycin stability in gastric juice using octanesulphonic acid has been reported [18]. However, none of these methods were able to determine decladinose clarithromycin.

To date, no suitable sensitive HPLC methods have been published that allow the simultaneous measurement of clarithromycin and its metabolites when only limited, small-volume biological samples are available (0.1 ml or less). In this paper, we describe a rapid, sensitive and selective HPLC assay for the simultaneous determination of clarithromycin, its 14-OH-clarithromycin metabolite and (for the first time) its decladinose acid degradation product in rat gastric juice aspirate, plasma and gastric tissue samples.

2. Experimental

2.1. Chemicals

Clarithromycin was a gift from Abbot Labs. (Abbot Park, IL, USA). 14(*R*)-Hydroxy-clarithromycin and omeprazole sodium were kindly donated (Astra Hässle, Mölndal, Sweden). Roxithromycin, sodium hydroxide and phosphate-buffered saline were purchased from Sigma (Poole, UK). Trisodium phosphate dodecahydrate, anhydrous potassium carbonate and sodium chloride were purchased from Fluka (Poole, UK). Disodium hydrogenphosphate dihydrate was obtained from Fisons (Loughborough, UK). Sodium dihydrogen orthophosphate dihydrate, hydrochloric acid, HPLC grade *n*-hexane, 2-butanol and acetonitrile were purchased

from Fischer (Loughborough, UK). Pentagastrin was bought from Cambridge Laboratories (Newcastle upon Tyne, UK). Most chemicals were of analytical grade or better. Water obtained from an Elga Maxima water purification system (Elga Ltd., High Wycombe, UK) was used. Blank rat plasma and blank rat stomach tissue were obtained from untreated rats.

Decladinose clarithromycin was synthesised by the acid-catalysed hydrolysis of clarithromycin. Clarithromycin (100 mg) was dissolved in 1.0 *M* hydrochloric acid (1.2 ml) and incubated at 37 °C for 30 min. The solution was then alkalinised by adding 1.0 *M* sodium hydroxide (1.4 ml) and repeatedly extracted (three times) by adding 2-ml aliquots of *n*-hexane/2-butanol (80:20). Decladinose clarithromycin was obtained in a 95% yield after evaporation of the solvents. The purity of decladinose clarithromycin was assessed by NMR, HPLC–ECD and electrospray ionisation LC–MS. The NMR spectrum obtained for the purified product was consistent with the previously published NMR spectrum for decladinose clarithromycin [5]. Both the HPLC–ECD and LC–MS showed a single peak in the chromatogram where the electrochemically active peak was identical to the peak detected with the mass spectrometer in TIC mode. Electrospray mass spectrometry yielded a major peak with a m/z value corresponding to the molecular ion of decladinose clarithromycin (m/z 590) and a minor peak (m/z 554) suggesting the loss of water caused by the ionisation process, as has been described before [5]. These results indicate that the synthesised decladinose clarithromycin had a purity of at least 95%.

2.2. Instrumentation

The HPLC system consisted of a Gilson 231XL automatic sample injector, Gilson 401 dilutor, an ESA 508 HPLC pump, a computer running 715 software (Gilson Medical Electronics, Villiers le Bel, France), and an ESA Coulochem II electrochemical detector fitted with an ESA 5020 guard cell (1000 mV), and an ESA 5011 analytical cell, set at a voltage of E1: 600 mV (screen voltage) and E2: 850 mV (ESA Analytical, Huntingdon, UK). A guard column (20×2.0 mm I.D.) packed with 5 μm Kromasil ODS material and a Kromasil ODS 5 μm (75×4.6 mm I.D.) (HiChrom, Reading, UK) analytical column were used. The column temperature was maintained at 30 °C using a model 7990 column oven from Jones Chromatography (Hengoed, UK). The mobile phase consisted of acetonitrile–aqueous phosphate buffer (pH 7.0, 0.086 *M*) (45:55 v/v). The mobile phase solvents were filtered through a 0.45-μm nylon or nitro-cellulose membrane filter (Whatman Ltd., Maidstone, Kent, UK), mixed, degassed using helium and equilibrated for 36 h by continuous recycling to remove residual electrochemical activity. The flow-rate was 1.0 ml min^{-1} and the sample injection volume was 20 μl.

2.3. Extraction procedure

To 50 μl of a plasma sample in an Eppendorf tube (2.0-ml capacity), 10 μl of the roxithromycin internal standard solution (25 μg ml^{-1}) and 150 μl of a pH 11 phosphate buffer (0.286 *M*) were added. The solution was vortex-mixed briefly and 1.0 ml of a *n*-hexane/2-butanol (4:1) mixture was added. The solution was then vortex-mixed for 5 min and centrifuged for 5 min at 2000 *g*. The aqueous layer was snap frozen by placing the tube in liquid nitrogen for exactly 10 s. The organic (top) layer was decanted into a clean Eppendorf tube and evaporated in vacuo for 40 min at 50 °C using a Jouan microcentrifuge evaporator (Jouan RC 10.22 concentrator/evaporator coupled to the RCT 120 cold trap and an Edwards 5 vacuum pump). The residue was reconstituted in 75 μl mobile phase, transferred onto an Eppendorf filter device (Vectaspin micro, 0.45-μm pore size polypropylene filter inserts) (Whatman Ltd., Maidstone, Kent, UK) and centrifuged at 2000 *g* for 5 min to remove undissolved particulate matter. The filtrate was analysed for clarithromycin content by HPLC.

The rat stomach was snap-frozen in liquid nitrogen and transferred into an polyethylene sample bag. The frozen stomach was then crushed while still frozen using a table-top vice to give a homogenous liquefied mass. This was then immediately re-frozen in the bag in liquid nitrogen, transferred into a pre-weighed Eppendorf tube (1.5-ml capacity) and weighed. Phosphate buffered saline (100 μl) was added to obtain a homogenised stomach tissue sample for each stomach analysed. Gastric aspirate (200 μl) was

treated in an identical manner to plasma. The extraction of homogenised stomach tissue was carried out twice by admixture and removal of an extra aliquot of *n*-hexane/2-butanol (4:1) (1.0 ml) to the tissue homogenate after the initial removal of the organic layer. The two organic layers obtained from the gastric tissue extract were subsequently pooled and treated identically to the extracts of the plasma and gastric aspirate samples.

2.4. Calibration and validation

Calibration samples of clarithromycin, 14-OH-clarithromycin and decladinose clarithromycin were prepared in saline (200 μl), blank plasma (50 μl) and HPLC mobile phase. Saline was used to sample the stomach contents in the rat pharmacokinetic experiments (see Section 2.5) and was therefore also used as a blank for gastric juice aspirate. Stomach tissue calibration samples were prepared by adding stomach tissue (0.25 g) from untreated rats to calibration solutions in saline. The gastric aspirate, plasma and stomach tissue calibrations were carried out over a clarithromycin concentration range of 0.10–20.3 μg ml^{-1}, a 14-OH-clarithromycin concentration range of 0.08–15.4 μg ml^{-1} and a decladinose clarithromycin concentration range of 0.06–12.5 μg ml^{-1} for both gastric juice aspirate and plasma. These concentration ranges were 0.51–40.6, 0.39–30.9 and 0.31–25.0 μg g^{-1}, respectively, for clarithromycin, 14-OH-clarithromycin and decladinose clarithromycin in gastric tissue.

The analyte/internal standard peak area ratios of clarithromycin, 14-OH-clarithromycin and decladinose were calculated and used to construct calibration lines of peak area ratio against drug concentration in gastric aspirate, plasma and stomach tissue using unweighted linear regression analysis. Slope, intercept and regression coefficient of the calibration lines were determined. The analyte recovery was calculated by comparing the peak area of the extracted samples to the peak area from the unextracted standard solutions of equivalent concentration prepared in mobile phase. Quality control samples of fixed concentration (Table 1) were prepared to determine the intra-day (six replicate analyses on a single day) and inter-day (six replicate analyses on six separate days) precision and accuracy.

The limit of detection was defined by the concentration of amoxicillin in the sample matrix giving a signal-to-noise ratio of 3:1. The lower limits of quantitation (LOQ) for the assays were defined from the linearity tests as the lowest concentration of the analytes to give an RSD of 20% or less for intra-day precision and accuracy.

2.5. Biological application of the methods: pharmacokinetic study in rats

Male Wistar rats, weighing 250–370 g were fasted for 24 h prior to experimentation. The stomach of the anaesthetised rat was cannulated to sample the gastric juice produced by securing a cannula into the antral part of the stomach via the duodenum and pylorus. The stomach was gently lavaged 4–6 times using this cannula with 1.5-ml aliquots of 0.9% saline until the aspirate was free of debris. A clean 1.5-ml aliquot of saline was introduced into the stomach and the abdominal wound was covered with moistened tissue. Gastric acid secretion was either suppressed by administering a single intravenous (i.v.) bolus of 20 μmol kg^{-1} omeprazole sodium dissolved in 0.9% saline via the tail vein, or stimulated by administering i.v. boluses of 25 μg kg^{-1} pentagastrin every 15 min.

The experiment was started by administering a 26 mg kg^{-1} i.v. bolus and starting an i.v. infusion (7.3 mg kg^{-1} h^{-1}) of clarithromycin. Blood plasma samples (0.3 ml) were taken from the carotid artery at 15-min intervals for 2 h. The complete gastric content was aspirated at the same time points. The stomach was removed and halved at the end of the experiment. Plasma, gastric juice aspirate and tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C pending analysis.

The presence of the clarithromycin decladinose acid degradation product in a real gastric juice aspirate sample from a rat was confirmed by collecting the peak running at the same retention time as the synthesised decladinose acid breakdown standard (see Section 2.1) and confirming its structure using mass spectrometry analysis.

Table 1
Intra- and inter-day precision and accuracy for the assay of clarithromycin, 14-OH-clarithromycin and decladinose clarithromycin: (a) gastric juice aspirate, (b) plasma and (c) gastric tissue

	Spiked ($\mu g\ ml^{-1}$)	Intra-assay ($n=6$)			Inter-assay ($n=6$)		
		Measured (mean±STD) ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)	Measured (mean±STD) ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)
(a) Gastric aspirate							
Clarithromycin	0.25	0.23±0.008	3.4	91.9	0.22±0.021	9.7	87.2
	1.02	1.00±0.019	1.9	98.4	1.00±0.044	4.4	98.8
	15.24	15.07±0.31	2.0	98.9	15.48±0.25	1.6	101.6
14-Hydroxy-clarithromycin	0.19	0.20±0.014	7.1	105.0	0.19±0.022	11.6	96.4
	0.77	0.75±0.024	3.2	97.5	0.73±0.046	6.3	94.9
	11.58	11.71±0.16	1.3	101.1	11.61±0.14	1.2	100.2
Decladinose	0.16	0.15±0.002	1.5	97.5	0.15±0.026	17.1	98.3
	0.62	0.60±0.005	0.9	95.7	0.59±0.015	2.6	94.3
	9.36	9.41±0.22	2.4	100.5	9.45±0.18	1.9	100.9
(b) Plasma							
Clarithromycin	0.25	0.24±0.018	7.7	94.9	0.22±0.032	14.3	88.4
	2.54	2.45±0.127	5.2	96.6	2.41±0.138	5.7	94.7
	15.24	15.68±0.36	2.3	97.1	15.26±0.21	1.4	100.1
14-Hydroxy-clarithromycin	0.19	0.18±0.016	8.6	94.9	0.18±0.022	12.2	91.6
	1.93	1.90±0.049	2.6	98.5	1.80±0.055	3.1	93.3
	11.58	11.73±0.23	2.0	101.3	11.59±0.10	0.9	100.1
Decladinose	0.16	0.15±0.006	4.1	93.3	0.15±0.033	21.6	96.4
	1.56	1.56±0.044	2.8	100.1	1.45±0.059	4.1	92.9
	9.36	9.54±0.15	1.6	101.9	9.39±0.09	1.0	100.3
(c) Gastric tissue[a]							
Clarithromycin	0.51	0.50±0.021	4.2	99.1	0.51±0.061	12.0	100.3
	2.03	2.06±0.055	2.7	98.7	1.98±0.067	3.4	97.6
	20.3	20.48±0.59	2.9	99.2	20.40±0.38	1.9	100.2
14-Hydroxy-clarithromycin	0.39	0.38±0.021	5.5	98.7	0.39±0.043	11.0	100.7
	1.54	1.58±0.052	3.3	97.4	1.57±0.17	10.7	101.6
	15.4	15.41±0.42	2.7	99.8	15.80±0.48	3.0	102.1
Decladinose	0.31	0.28±0.018	6.3	90.7	0.29±0.033	11.3	93.5
	1.25	1.17±0.028	2.4	93.7	1.19±0.081	6.8	95.2
	12.5	12.10±0.31	2.5	96.9	12.70±0.26	2.0	101.4

[a] Gastric tissue concentrations in $\mu g\ g^{-1}$.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of blank gastric juice aspirate, plasma and stomach tissue and for samples containing clarithromycin are shown in Fig. 2. The retention times for clarithromycin, 14-OH-clarithromycin, decladinose clarithromycin and that of the internal standard, roxithromycin, were 5.9, 2.2, 2.0 and 6.6 min, respectively. No interfering peaks were noticeable in the chromatograms of blank gastric juice aspirate, plasma and gastric tissue. Table 1 summarises the accuracy and reproducibility of the assay methods.

Eleven-point calibration lines were used to validate the assay in gastric juice aspirate and plasma and a nine-point calibration line was used for gastric tissue over the ranges outlined above. The lower limits of quantitation, the recovery after extraction and the corresponding correlation coefficients (r^2) are represented in Table 2 and confirm the linearity

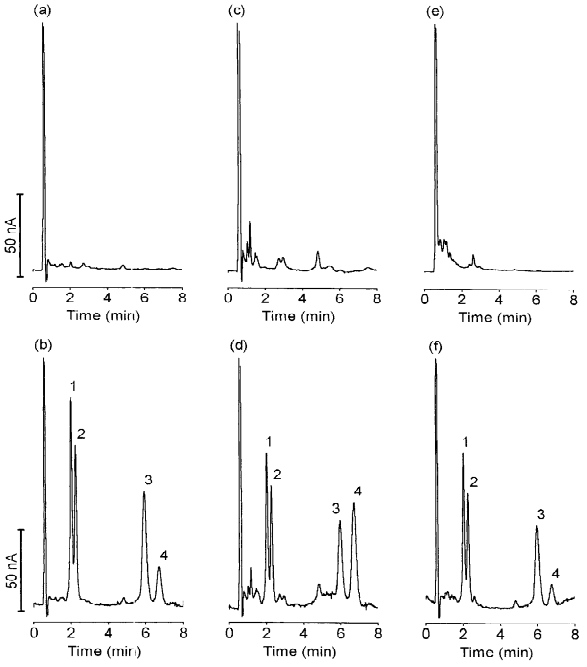

Fig. 2. Chromatograms of extracts of: (a) blank gastric aspirate, (b) spiked gastric aspirate, (c) blank plasma, (d) spiked plasma, (e) blank gastric tissue and (f) spiked gastric tissue. Samples were spiked with decladinose (6.24 μg ml^{-1} or μg g^{-1}, peak 1), 14-OH-clarithromycin (7.72 μg ml^{-1} or μg g^{-1}, peak 2), clarithromycin (10.2 μg ml^{-1} or μg g^{-1}, peak 3) and the internal standard roxithromycin (peak 4).

of the assay. Exhaustive extraction of the tissue homogenates indicated that 87±2% of the total amount that could be recovered was extracted after the first liquid–liquid extraction and 94±2% ($n=5$) after the second extraction. The absolute recoveries ranged from 78 to 113%, compared to unextracted clarithromycin, 14-OH-clarithromycin and decladinose clarithromycin standards. The recoveries measured in the gastric tissue samples included a small, but unquantifiable volume change during the centrifugation/filtration step to remove the undissolved particulate matter and this may explain the reported recoveries of greater than 100%.

We observed a small but variable adsorption of clarithromycin (at low concentrations) to glassware during the extraction process (data not shown) which has not been reported in previous assays, perhaps because of the lower concentrations measured in our procedures. This unwanted adsorption could be prevented by silanising the glassware, or by carrying out the extraction in polypropylene Eppendorf vials.

3.2. Identification of decladinose clarithromycin in rat gastric juice

The sample of the peak collected from gastric juice aspirate with the retention time of 2.0 min was

Table 2
Inter-assay calibration linearity, slope, recoveries and limits of quantification of the assay for clarithromycin, 14-OH-clarithromycin and decladinose clarithromycin in rat gastric aspirate, plasma and gastric tissue

	(r^2)	RSD% of slope	Recovery (%)	LOQ (μg ml^{-1})
Gastric aspirate				
Clarithromycin	0.9995±0.0002	7.7	94.2±12.6	0.15
14-OH-clarithromycin	0.9994±0.0004	6.0	77.8±8.6	0.08
Decladinose	0.9994±0.0002	7.3	81.5±9.4	0.06
Plasma				
Clarithromycin	0.9992±0.0005	4.3	93.5±22.3	0.10
14-OH-clarithromycin	0.9996±0.0001	3.0	84.8±10.1	0.08
Decladinose	0.9995±0.0002	4.8	88.7±14.5	0.16
Gastric tissue[a]				
Clarithromycin	0.9993±0.0003	10.5	113.0±17.0	0.51
14-OH-clarithromycin	0.9991±0.0006	19.9	100.0±11.7	0.39
Decladinose	0.9995±0.0003	16.5	105.1±14.2	0.31

All means are the average of at least six replicates.
[a] LOQ in μg g^{-1}.

confirmed to be decladinose clarithromycin by electrospray ionisation LC–MS. The major molecular ion from the sample was found to be m/z 590 and a minor peak was found at m/z 554, identical to the fragmentation pattern of the synthesised decladinose clarithromycin standard (see Section 2.1). As far as we are aware, this is the first time that the presence of decladinose clarithromycin has been confirmed in either gastric juice or gastric tissue.

3.3. Biological application: pharmacokinetic study in rats

The developed and validated assay method was used to measure the concentrations of clarithromycin, 14-OH-clarithromycin and decladinose clarithromycin in rat gastric juice, plasma and gastric tissue following the i.v. administration of clarithromycin. Typical chromatograms obtained from this rat pharmacokinetic study are shown in Fig. 3. In gastric juice, clarithromycin and decladinose clarithromycin were present but little or no 14-hydroxy-clarithromycin was detected. Clarithromycin was readily quantified in plasma but the 14-OH-clarithromycin metabolite, although detected, was below its limit of quantification and the decladinose clarithromycin could not be detected in plasma. In gastric tissue it was possible to quantify all three of the compounds.

Fig. 4 shows a typical concentration–time profile of clarithromycin and decladinose clarithromycin in gastric juice aspirate and in plasma following i.v. administration of clarithromycin. The clarithromycin concentration was lower in gastric aspirate than in plasma throughout the experiment.

It was readily possible to measure the tissue concentrations of clarithromycin and decladinose clarithromycin at all stages of the rat pharmacokinetic experiment; at the end of the experiment measured concentrations in rat gastric tissue were found to be 13.0 and 0.4 μg g^{-1}, respectively.

This method been applied to the detection of clarithromycin and its metabolites in human gastric juice, plasma and gastric biopsy samples (typical

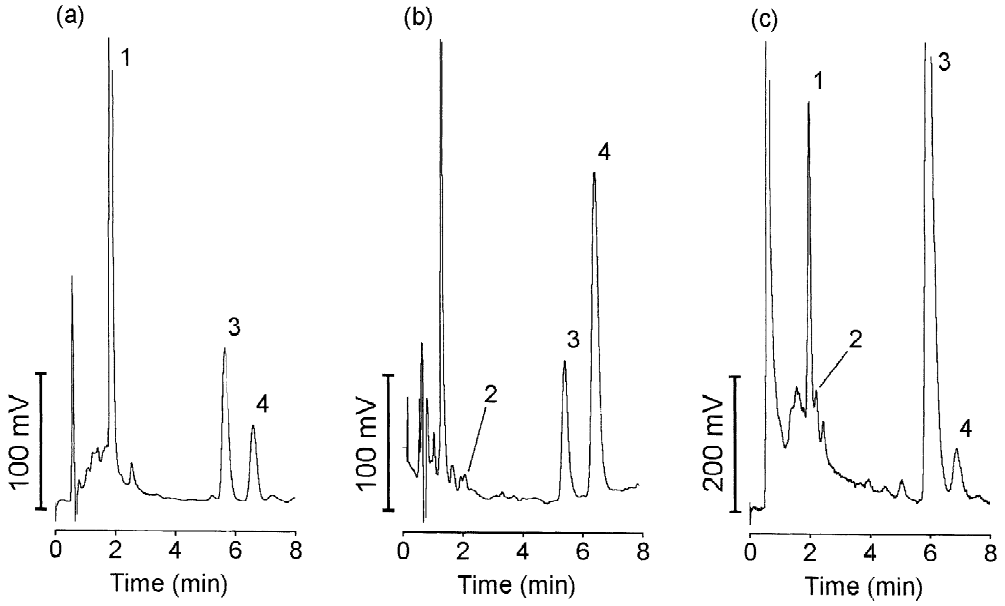

Fig. 3. Chromatograms of samples from a rat given an i.v. infusion of clarithromycin: (a) gastric aspirate (clarithromycin 4.67 μg ml^{-1}, decladinose clarithromycin 13.19 μg ml^{-1}), (b) plasma (clarithromycin 5.08 μg ml^{-1}, 14-OH-clarithromycin trace below LOQ); and (c) gastric tissue (clarithromycin 14.0 μg g^{-1}, decladinose clarithromycin 1.59 μg g^{-1}, 14-OH-clarithromycin trace below LOQ). Peak 1 = decladinose clarithromycin; peak 2 = 14-OH-clarithromycin; peak 3 = clarithromycin and peak 4 = internal standard roxithromycin.

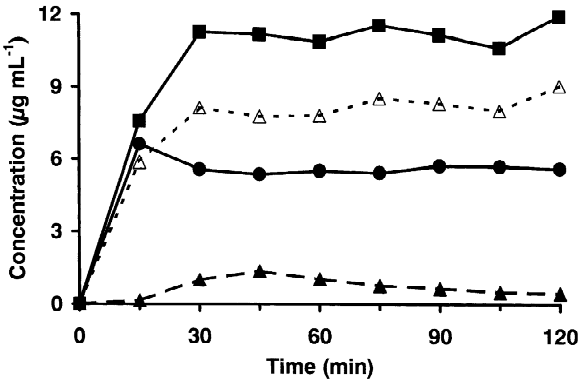

Fig. 4. Typical clarithromycin and decladinose clarithromycin concentration versus time profiles for gastric aspirate and plasma from a rat treated with pentagastrin: clarithromycin concentration in gastric aspirate (▲) and plasma (■), decladinose clarithromycin concentration in gastric aspirate (●) and the total clarithromycin (degraded and undegraded) concentration in gastric aspirate (△).

weight 5–10 mg, data not shown). More than 2500 samples have been successfully analysed in total.

4. Conclusion

We have developed a robust coulometric-based HPLC assay for the simultaneous determination of clarithromycin, its 14-OH-clarithromycin metabolite and its decladinose acid breakdown product in small volumes of rat gastric juice aspirate, plasma and gastric tissue. The short analysis time, high sensitivity and reproducibility of the method were demonstrated in a biological application involving pharmacokinetic studies of i.v. administered clarithromycin in individual rats. We have also confirmed, for the first time, the presence of decladinose clarithromycin in rat gastric juice and gastric tissue and shown that this acid breakdown product of clarithromycin can be quantified in gastric juice and tissue.

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