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DETERMINATION OF Ro 14-1761, A NEW THIRD-GENERATION CEPHALOSPORIN, IN THE PLASMA AND MILK OF CATTLE BY COLUMN SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific high-performance liquid chromatographic procedure was developed for the determination of the third-generation cephalosporin Ro 14-1761 in cow plasma and milk. The molecular structure of the new antimicrobial was very close to that of ceftriaxone, but the high-performance liquid chromatographic methods available for the latter could not be used as Ro 14-1761 adsorbed and/or degraded during the chromatographic process. Furthermore, the high-performance liquid chromatographic technique derived for ceftriaxone was not sensitive enough for our purposes. In the new assay, the plasma (milk) protein was precipitated with acetonitrile after dilution of the sample with water. For low concentrations ($\leqslant 10$ μg/ml), the supernatant obtained after centrifugation was concentrated by extracting acetonitrile with methylene chloride. Quantification was performed by column switching high-performance liquid chromatography with UV detection (274 nm) using ion-pair reversed-phase chromatography. Ethylenediaminotetraacetic sodium salt had to be added to the mobile phase (1.2 m*M*) to prevent adsorption and/or degradation of the cephalosporin on the analytical column. The selectivity of the chromatographic separation was enhanced by heating the column to *ca.* 50°C.

The drug recovery was better than 85%. The limit for quantitative determination in both milk and plasma was 0.1 μg of Ro 14-1761 per millilitre with an accuracy of 1% (coefficient of variation 10%). The overall accuracy and precision were 1–10% in the 0.1–100 μg/ml concentration range.

INTRODUCTION

Ro 14-1761, whose systematic name is (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-acetamido]-3-{[(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-*as*-triazin-3-yl)thio]-methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene -2-carboxylic acid, is a member of the "third-generation" cephalosporin antimicrobials. It is characterized by a relatively broad antibacterial spectrum, including good activity against streptococci, staphylococci and escherichiae strains, together with high resistance to beta-lactamase-producing organisms. These characteristics offer potential clinical advantages,

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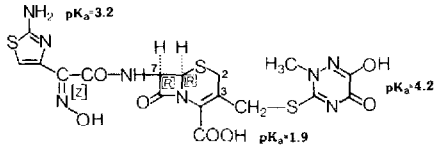
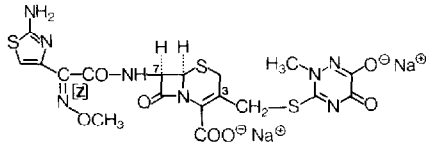


Ro 14-1761



Ceftriaxone

Fig. 1. Molecular structures of Ro 14-1761 and ceftriaxone.

which may lead to the use of this drug in treating mastitis infections in cattle. The molecular structure of Ro 14-1761 is very close to that of ceftriaxone: the oxime in the side-chain at the C-7 position of the beta-lactam moiety bears a methyl group in ceftriaxone, whereas it is free in Ro 14-1761 (Fig. 1). Currently published methods for the determination of ceftriaxone and other cephalosporins in biological fluids include bioassay[1–4] and high-performance liquid chromatography (HPLC)[1,5,6]. The bioassay tends to be cumbersome and is susceptible to interference from drug metabolites and currently administered antimicrobial drugs. HPLC appears to be more specific. Two of the HPLC methods utilized the technique of ion-pair reversed-phase chromatography[1,5], whereas the third used a normal phase column[6]. Unfortunately, these methods appeared to be inadequate to quantify Ro 14-1761 in cow plasma and milk. When using reversed-phase methods, strongly distorted peaks or split peaks were observed; likewise, Ro 14-1761 could not be eluted using a normal phase system such as the one described by Ascalone and Dal Bò[6].

In this report we describe a reversed-phase HPLC method using ion-pair chromatography with column switching for the quantification of Ro 14-1761 in cow plasma and milk.

EXPERIMENTAL

Reagents, solvents and materials

Ro 14-1761 was kindly supplied by Dr. F. Furlenmeier (Hoffman-La Roche) as its disodium (Ro 14-1761/001) or N,N′-dibenzylethylenediamine (Ro 14-1761/003) salts. Acetonitrile was HPLC grade from Rathburn (U.K.); methylene chloride, ethanol, and ethylenediaminetetraacetic disodium salts (Titriplex® III) were all p.a. grade from E. Merck (Darmstadt, F.R.G.). *Trans*-1,2-Diamino-cyclohexane-N,N,N′,N′-tetraacetic acid and tetraoctylammonium bromide were p.a. grade from Fluka (Buchs, Switzerland). The buffer solution was the ready-to-use titrisol from Merck. Water was distilled twice in an all-glass apparatus.

Standard solutions; plasma and milk solutions

Typically, a stock solution of 4.831 mg of Ro 14-1761/001 or /003 in 10 ml of water buffered to pH 8 was prepared. This solution was then used to prepare plasma or milk standards by adding known volumes to blank milk or blank plasma obtained from oxalated cow blood. Control plasma and milk solutions were prepared by successive dilutions of a 96.62 μg/ml standard solution of the drug in plasma (milk). Both the stock solution and the control plasma/milk were kept at +4°C and prepared again after one week. All the calculations were carried out using the free acid Ro

14-1761 concentration after a correction factor was applied to the weighed sodium or N,N'-dibenzylethylenediamine salt.

Sample preparation procedure

Doubly distilled water (2 ml and 6 ml for plasma and milk, respectively) was added to 1 ml of plasma (or 3 ml of milk), followed by 6 ml of acetonitrile (or 18 ml for milk). After vortex mixing, the plasma (milk)–acetonitrile mixture was shaken for 5 min using a rotating shaker (Heidolph, F.R.G.) and then centrifuged for 5 min at 3000 rpm (1800 *g*, 10°C). The supernatant (20 μl) was injected for HPLC analysis.

When the plasma or milk concentration of Ro 14-1761 was lower than 10 μg/ml, the procedure was the same as above and was followed by a concentration step: after centrifugation, 6 ml of the supernatant were transferred to a clean tube and 6 ml of methylene chloride were added. The mixture was shaken for 5 min using a rotating shaker. After centrifugation (5 min, 1200 *g*), the aqueous phase was separated, and the remaining trace of solvent was removed under vacuum (200 Torr, 5 min, 30°C). Then 20–40 μl was injected onto the HPLC column.

Chromatographic procedure

Apparatus design and operating conditions. The HPLC apparatus consisted of a Kontron LC 414 pump (Kontron, Zurich, Switzerland), an autoinjector (Kontron, Model ASI 45), a 125 × 4 mm I.D. stainless-steel column filled with MOS Hypersil C_8 5 μm (Shandon, Frankfurt (M), F.R.G.), and a Spectroflow 773 LC-UV detector (Kratos, Riehen, Switzerland) connected to a Spectra Physics SP 4100 computing integrator (Spectra Physics, Basle, Switzerland). The columns were packed according to the method of Halàsz *et al.*[7,8]. When in operation, the column was heated by means of a column block heater (Ercatech, Berne, Switzerland). The mobile phase was a mixture of 300 ml of acetonitrile containing 1 g of tetraoctylammonium bromide ("TOAB", Merck), 10 ml of ethanol, 12 ml of buffer solution of ethylenediaminetetraacetic disodium salt (EDTA 0.1 *M*, pH 8), made up to 1 l with doubly distilled water.

Because concentrated biological samples were injected, the analytical column was protected from highly retained endogenous products by means of a 4 × 4 mm I.D. Hibar LiChrocart (LiChrosorb RP-18) pre-column[9] filled with RP-18 LiChrosorb (Merck). A short time after the injection (less than 1 min), the retained plasma products were washed from the pre-column by backflushing. The additional apparatus for column switching consisted of a second pump (414T, Kontron), and an electronic interface (designed and prepared in the PF/BP electronic workshop, Hoffmann-La Roche, Basle, Switzerland) for actuating the automatic switching valve (Rheodyne, Coteti, CA, U.S.A. Model 7000 HPLC switching valve; Model 7001 pneumatic actuator, Model 7163 solenoid valve) which directed the flow from the pre-column either to the analytical column or to waste. The interface was controlled by the integrator. Fig. 2 is a schematic of the complete HPLC system.

The operating conditions were as follows: flow-rate, 1.5 ml/min; column temperature, 50°C; detection wavelength, 274 nm; volume injected (fixed loop), 20 μl for concentrations higher than 10 μg of Ro 14-1761 per millilitre, and 40 μl for lower concentrations. Under these conditions, the retention time of Ro 14-1761 was *ca.* 8 min.

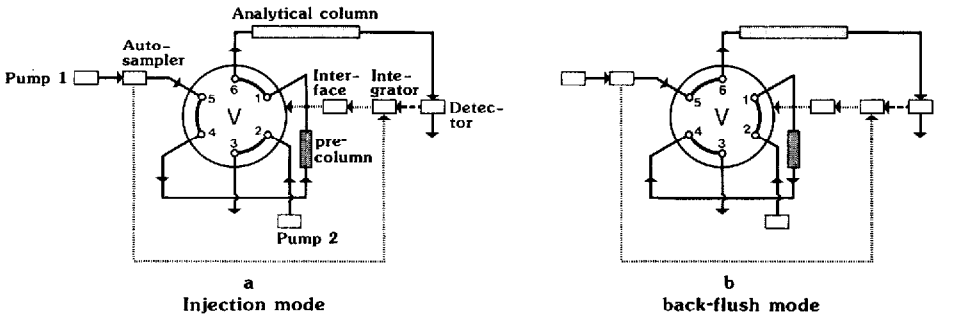


Fig. 2. Design of the column switching HPLC apparatus in (a) injection mode and (b) back-flush mode. The solid lines show the solvent flow, the dashed line shows the analogue signal from the detector, and the dotted line the electronic control of switching valve.

Chromatographic process description (Fig. 2a and b). The supernatant obtained after plasma (milk) protein precipitation, or the aqueous phase remaining after the extraction of the supernatant with methylene chloride, was injected by the autosampler onto the pre-column (Fig. 2a). Immediately after the elution of Ro 14-1761 from the pre-column (0.80 min), the HPLC valve (V) was switched to the second position (Fig. 2b) by means of the computing integrator, and the cephalosporin was then chromatographed in the usual manner on the analytical column, while longer-retained endogenous products were removed from the system by back-flushing the pre-column. When chromatography was complete, the valve (V) was switched back to the initial position (Fig. 2a). After a 2-min equilibration time, the system was ready for the next run. A complete cycle lasted *ca.* 13 min.

Calibration and calculation

The calibration samples, each consisting of nine plasma standards containing the appropriate concentration of Ro 14-1761, were processed as described above. The calibration curves were obtained by least-squares regression of the peak height of Ro 14-1761 against the respective concentrations. These curves were then used to interpolate the concentrations of the cephalosporin from the respective peak height of quality control or unknown samples. All data processing and calculations were carried out by the Spectra Physics computing integrator and the minifile 4100D.

RESULTS AND DISCUSSION

Chromatographic system

Initial evaluation indicated that simple reversed-phase HPLC techniques employed for other cephalosporins did not provide acceptable chromatographic peak shape or retention of Ro 14-1761. The single ion-pairing HPLC procedures reported for ceftriaxone[1,5,6,10] resulted in either no retention or strong peak assymetry. Various alkyl ammonium and sulphonate salts with C_4–C_{18} alkyl chain, as well as different reversed-phase materials, were tested at various pH values (pH 4 to 8) without any success. With the ammonium salts, distorted peaks and even peak splitting (depending on the Ro 14-1761 concentration) were sometimes observed. This was at-

tributed to the involvement of two discrete chromatographic mechanisms operating simultaneously in the column[11–13].

Mobile phase composition and working temperature. A combination of tetraoctylammonium bromide and methylamine reagent in acetonitrile–water (1:1) at pH 8 gave some retention of the cephalosporin, with acceptable peak shape after the mobile phase was recycled overnight on the column. Other amines such as mono-, di-, triethanolamines, and mono-, di-, triethylamines gave poor peak shapes. Increasing the column temperature did not improve these results.

Such distorted chromatographic peaks could result from irreversible or slowly reversible adsorption and/or degradation on the surface of the reversed-phase material. These phenomena could be catalysed by metal ions. Surprisingly, ceftriaxone was eluted with good peak shape in almost all of the investigated chromatographic systems. The difference in molecular structure between ceftriaxone and Ro 14-1761 is the oxime function in the C-7 side-chain of the beta-lactam moiety, which bears a methyl group in ceftriaxone, whereas it is free in Ro 14-1761. Such a free oxime group could react with metal cations, *e.g.* aluminium, which may be present on the surface of the solid support. EDTA was then tried as a complexing agent.

Changing from the phosphate buffer solution (pH 8) to a buffer prepared with EDTA disodium salt and potassium hydroxide (pH 8) resulted in a good peak shape (see Fig. 3). The chromatographic improvement was observed within a few minutes following the addition of the EDTA solution to the mobile phase. Such an effect had

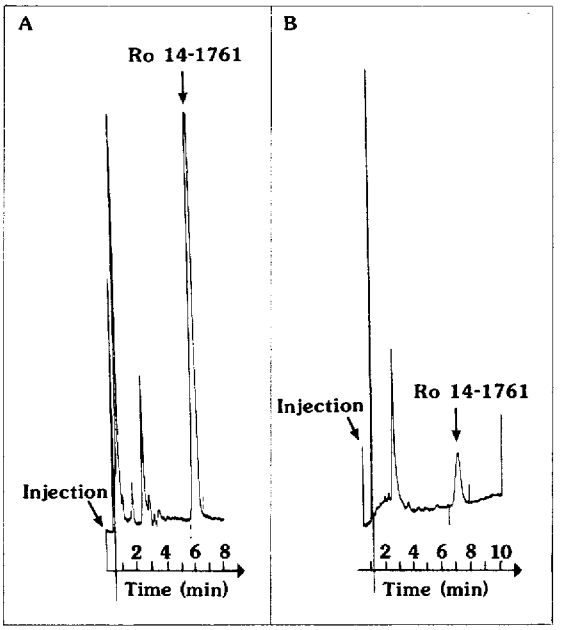


Fig. 3. Influence of EDTA on the peak shape and height of Ro 14-1761. Column, 125 × 4 mm I.D. MOS Hypersil (5 μm); mobile phase, 300 ml of acetonitrile, 1 g of TOAB, 10 ml of ethanol, buffer solution, and water to 1000 ml; flow-rate 1.5 ml/min. (A) Buffer solution, 12 ml of 0.1 *M* EDTA (pH 8); (B) buffer solution, 0.6 ml of phosphate buffer titrisol (pH 8). The injected solution contained 10 μg Ro 14-1761 per millilitre of water.

already been observed with cefpimizole by Lakings and Wozniak[14]. As ceftriaxone and Ro 14-1761 have the same UV spectrum (Fig. 4), the chromatographic peak heights of both substances should be the same at the same free acid concentration. As the two cephalosporins were separated in this system, this mixture could be used as an index to establish whether Ro 14-1761 was quantitatively eluted from the analytical column. Thus, it was observed that Ro 14-1761 could not be eluted quantitatively, although increasing the EDTA concentration did improve the results. Using *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid instead of EDTA led to similar observations.

Increasing the salt concentration decreased the selectivity of the separation with respect to the plasma (milk) endogenous compounds. Changes in retention time and a clear improvement of the selectivity were observed with the addition of 10 ml of ethanol to the mobile phase and when working at higher temperatures[15,16]. Fig. 5 shows how the chromatographic profile changed with the column temperature.

Influence of the reversed-phase material. Several reversed-phase materials, and various batch numbers within the same material were tested. The best chromatographic results were achieved with MOS Hypersil (5 μm). Nucleosil C_{18} or C_8 (5 μm), and ODS Hypersil C_{18} (5 μm) also gave good results, whereas other stationary phases such as μBondapak, LiChrosorb RP-18 or RP-8, Partisil 5, ODS-3 from Whatmann, Ultrasphere IP (Altex), were not adequate for Ro 14-1761 analysis.

Moreover, when the column was changed, it was necessary to tune the column temperature and the mobile phase composition to achieve the best chromatographic separation.

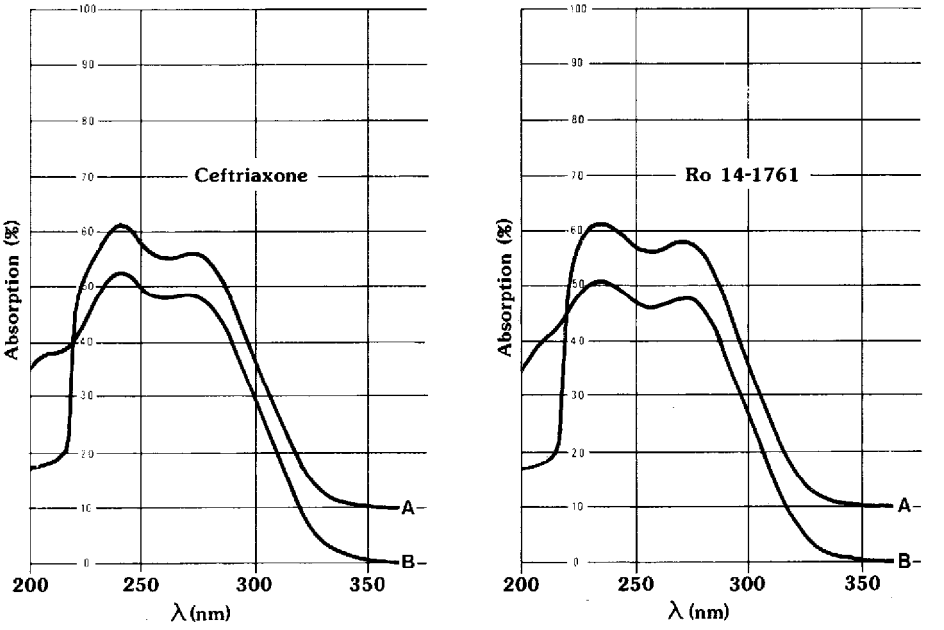


Fig. 4. UV spectra of ceftriaxone and Ro 14-1761. The compounds were dissolved in the mobile phase used for the HPLC analysis of Ro 14-1761, (A) with and (B) without tetraoctylammonium bromide. Cephalosporin concentration, 0.08 mol/l.

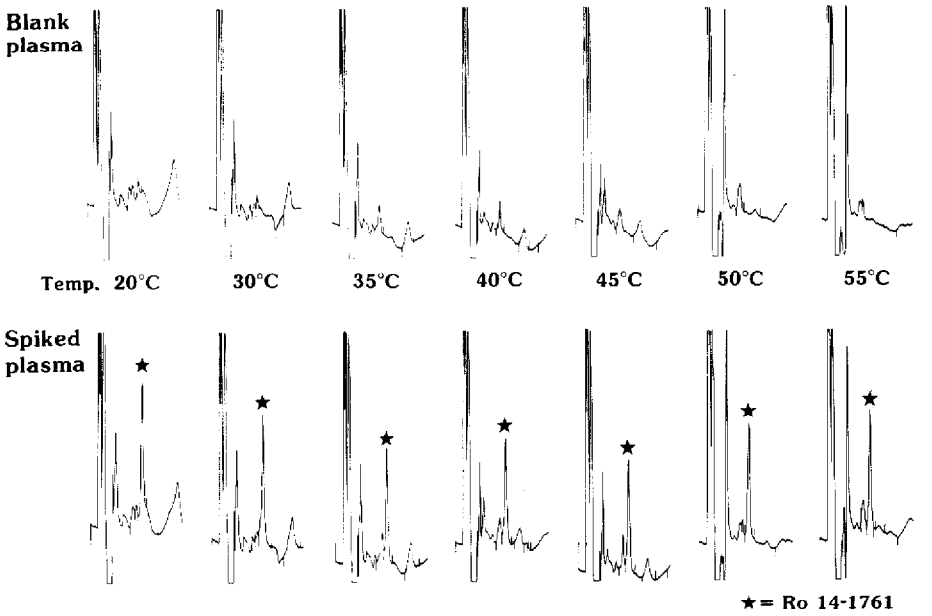


Fig. 5. Influence of the column temperature on the separation of Ro 14-1761 and endogenous compounds from plasma. The plasma samples were spiked with 10 μg/ml (20, 30 and 40°C) and 5 μg/ml (45, 50 and 55°C) of cephalosporin. Column: 125 × 4 mm I.D. MOS Hypersil (5 μm); mobile phase, 300 ml of acetonitrile, 1 g of TOAB, 10 ml of ethanol, 12 ml of 0.1 *M* EDTA (pH 8), and water to 1000 ml; flow-rate, 1.5 ml/min.

Sample preparation and recovery

The recovery of the drug was estimated from the change in the peak height when Ro 14-1761 was added to plasma or milk before protein precipitation, compared with the peak height when the drug was added to the final extract of a blank plasma (milk). During this study, it was observed that Ro 14-1761 was adsorbed onto glass to some extent when an aqueous solution of the cephalosporin was used.

The recovery of Ro 14-1761 in plasma and milk was 85–90% and 93–98%, respectively (Table I).

Selectivity

Ro 14-1761 has a high polarity which makes it impossible to extract the drug into an organic solvent. Therefore, sample preparation must involve protein precipitation and direct injection of "dirty" biological samples containing large amounts of endogenous products, which may interfere with the assay. Several blank plasma and milk samples from cow were analysed as described in the previous section. The assay is specific for Ro 14-1761 down to 10 μg of Ro 14-1761 per millilitre without heating the analytical column. Below this limit, the plasma (milk) endogenous products, which eluted together with the cephalosporin, disturbed the assay. This was overcome by heating the analytical column to *ca.* 50°C (see Fig. 5). The temperature had to be adapted from column to column, even when the reversed-phase material was from the same batch. Small changes in the temperature (± 5°C) strongly influenced the quality of separation.

TABLE I

RECOVERY OF Ro 14-1761 FROM PLASMA AND MILK

$n = 5$.

Medium	*Conc. added (μg/ml)*	*Recovery (%)*
Plasma	25	89.7
	100	86.1
	150	89.4
Milk	25	98.1
	100	92.9
	150	96.3

When the system was optimized, the assay was specific in the 0.1–100 μg/ml concentration range.

Linearity and limit of quantitation

According to the expected concentration in the unknown or quality control samples, two calibration ranges were used, either 0.1–1 μg/ml of Ro 14-1761 or 10–150 μg/ml. A linear relationship between the peak height and the concentration was obtained within both these ranges. The values of the slope, the intercept, and the coefficient of determination calculated from the calibration curves of Ro 14-1761 are listed in the Tables II and III. They were obtained on several consecutive days with plasma and milk, respectively. The limit for quantitative determination, defined as the concentration of Ro 14-1761 in plasma (milk) which can be measured with a precision and an accuracy better than 10%[17–19], was 0.1 ± 0.01 μg/ml ($n = 6$).

Precision and accuracy

The precision[20] of this method was evaluated over the concentration range 0.1–100 μg/ml plasma (milk). The overall intra-assay precision, determined by analysing each concentration at least five times on the same day, was found to be 1–10% in plasma (Table IV). The overall inter-assay precision determined over 5–7 consecutive days was better than 1–5% (Table V). The accuracy[20] was better than 5%

TABLE II

MEAN VALUE OF THE INTERCEPT (A), THE SLOPE (B), THE COEFFICIENT OF DETERMINATION (r^2), AND THE MEAN DEVIATION AROUND THE CURVE, CALCULATED FROM THE CALIBRATION LINES OF Ro 14-1761 OBTAINED ON FIVE CONSECUTIVE DAYS IN PLASMA

Coefficient	*Mean ± S.D.*	*Median*	*95% conf. limits*
A (μg/ml)	−69.2 ± 136.5	−113.8	−238.6/100.2
B	136.9 ± 9.1	133.9	125.6/148.1
r^2	0.9996 ± 0.0002	0.9997	0.9994/0.9999
Mean dev. (%)	1.6 ± 0.9	1.1	0.5/2.7

TABLE III

MEAN VALUE OF THE INTERCEPT (A), THE SLOPE (B), THE COEFFICIENT OF DETERMINATION (r^2), AND THE MEAN DEVIATION AROUND THE CURVE, CALCULATED FROM THE CALIBRATION LINES OF Ro 14-1761 OBTAINED ON SEVEN CONSECUTIVE DAYS IN MILK

Coefficient	*Mean ± S.D.*	*Median*	*95% conf. limits*
A (μg/ml)	−84.2 ± 114.5	−33.8	−190.1/21.8
B	187.01 ± 22.79	195	165.93/208.10
r^2	0.9997 ± 0.0003	0.9997	0.9994/1.0000
Mean dev. (%)	1.1 ± 0.4	1.1	0.7/1.4

in both cases. It was observed that the measured concentrations were always under the concentrations calculated from the weighted Ro 14-1761 added to the standard (*i.e.* the accuracy was always negative). This could originate from (i) an adsorption of the cephalosporin on the glass tube used during the sample preparation and/or (ii) a non-quantitative elution of the drug from the analytical column.

Stability of the new cephalosporin in milk

Solutions of Ro 14-1761 were processed in control plasma at concentrations

TABLE IV

Ro 14-1761: INTRA-ASSAY PRECISION AND ACCURACY IN PLASMA

Conc. added (μg/ml)	*Conc. found ± S.D. (μg/ml)*	*Accuracy: found − added (%)*	*95% conf. limits (μg/ml)*	*n*
0.1	0.100 ± 0.01	⩽1	0.093/0.196	6
1.0	0.985 ± 0.032	−1.5	0.962/1.007	10
24.15	24.10 ± 0.26	⩽1	23.8/24.4	5
96.62	96.54 ± 1.5	⩽1	94.7/98.4	5

TABLE V

Ro 14-1761: INTER-ASSAY PRECISION AND ACCURACY IN PLASMA

Conc. added (μg/ml)	*Conc. found ± S.D. (μg/ml)*	*Accuracy: found − added (%)*	*95% conf. limits (μg/ml)*	*n*
21.43	20.5 ± 0.4	−4.3	20.0/21.0	5
24.15	22.9 ± 0.9	−5.2	22.1/23.7	7
85.72	84.6 ± 0.8	−1.3	83.6/85.6	5
96.62	94.2 ± 1.0	−2.5	93.3/95.1	7

TABLE VI

Ro 14-1761: STABILITY IN MILK AT ROOM TEMPERATURE OVER 24 h

Determined according to the method of Timm *et al.*[21]. LL = lower limit; UL = upper limit; *D* = percentage difference in response after storage.

Conc. added (μg/ml)	*Peak height ref. 21*	*Peak height 24 h*	*D (%)*	*LL (%)*	*UL (%)*
10	1653	1436	−13	−16.2	−9.9
125	20 843	19 122	−8	−10.7	−5.6
250	41 056	37 176	−9	−11.7	−7.2

of 10, 125 and 250 μg/ml. They were stored at room temperature for 24 h under normal laboratory lighting conditions and analysed. Together with each set of stored samples, an equal number of freshly spiked samples were analysed to provide the 100% values. The procedure and subsequent statistical calculations were carried out according to the method of Timm *et al.*[21]. The data from these stability determinations are presented in Table VI. The results showed that Ro 14-1761 was not stable in milk at room temperature as a decrease of the peak height of *ca.* 10–16% was observed. Therefore, the plasma and milk samples containing the cephalosporin Ro 14-1761 should not be stored at room temperature for 24 h but should be deep frozen soon after collection (biological samples) or preparation (standard). Nevertheless, the assay may be regarded as suitable for pharmacokinetics in cow and residue evaluation in the milk following Ro 14-1761 administration.

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