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Determination of the cephalosporin antibiotic cephradine in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple and sensitive high-performance liquid chromatographic method, for the determination of cephradine in human plasma samples has been developed and validated. Cephradine and cephaloridine (internal standard) were extracted from human plasma by perchloric acid protein precipitation followed by centrifugation. Aliquots of the extracts were analysed by reversed-phase high-performance liquid chromatography (HPLC) utilising a polymeric reversed-phase PLRP-S column, followed by ultraviolet detection at 260 nm. The method has a working dynamic range from 0.2 to 30.0 μg/ml from 200 μl human plasma. The precision of the method at 0.2 μg/ml was 4.9% (intra-assay) and negligible (inter-assay) as calculated by one-way analysis of variance and the accuracy of the method at 0.2 μg/ml was −4.1% in terms of percentage bias. This method has been successfully applied to clinical studies including an oral bioequivalence study comparing the pharmacokinetics of 500 mg tablets of Kefdrin with 500 mg tablets of Velosef in healthy human volunteers.

Keywords: Cephalosporins; Cephradine

1. Introduction

Cephradine, 7-[α-D-(cyclohexa-1,4-dienyl)-glycylamino]-3-methyl-3-cephem-4-carboxylic acid, is a first generation cephalosporin, originally isolated in 1948. It has broad spectrum antibacterial activity against gram-positive and gram-negative microorganisms, through inhibition of bacterial cell wall synthesis. Cephradine is useful for treatment of infections of the urinary and respiratory tract, skin and soft tissues. Although cephradine has largely been superseded by second and third generation cephalosporins it nevertheless remains an important antibiotic in the developing world. Our bioanalytical strategy was to develop a simple high-performance liquid chromatography–UV detection (HPLC–UV) method with the required sensitivity, accuracy and precision to support bioequivalence studies with 500 mg oral doses of cephradine. HPLC–UV was the method of choice because of the need to be able to readily transfer the method developed to laboratories in other countries in order to support local operating company studies with cephradine. Also the need to automate the bioanalytical method was considered in

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order to improve on sample throughput whilst keeping equipment and labour costs low.

Poor reproducibility is often a failing of octadecylsilane (ODS, C_{18}) bonded HPLC columns under very low pH conditions [1,2]. However this type of reversed-phase column is widely recommended as the column of choice for chromatographing β-lactam antibiotics [3–8].

In order to improve chromatographic reproducibility at low pH a bioanalytical method was developed and validated. This utilises perchloric acid (pH 1) to precipitate proteins from human plasma samples and combines this with isocratic HPLC separation of the supernatants on a polymer column with poly(styrene–divinylbenzene) [9,10] as the stationary phase.

Cephaloridine, a structural analogue of cephradine was chosen as the internal standard in this method as it gave a similar UV response but was chromatographically resolved from cephradine and gave linear calibration lines of cephradine/cephaloridine response ratio versus concentration over the desired dynamic range.

This paper describes the bioanalytical method development, validation and its application to the determination of cephradine in human plasma samples from an oral bioequivalence study comparing the pharmacokinetics of Kefdrin and Velosef in 500 mg cephradine tablets in healthy human volunteers.

2. Experimental

2.1. Materials

Cephradine is a product of GlaxoWellcome (European Clinical Supplies, Stevenage, UK). Cephaloridine, (7*R*)-3-(1-pyridiniomethyl)-7-[(2-thienyl)-acetamido]ceph-3-em-4-carboxylate, used as the internal standard was purchased from Sigma (Gillingham, UK). Ammonium dihydrogen orthophosphate, orthophosphoric acid (85%, v/v) and perchloric acid (98%, v/v), all AR-grade, were obtained from BDH (Poole, UK). Acetonitrile and water, both HPLC-grade, were obtained from Rathburn (Walkerburn, UK).

2.2. Apparatus

The HPLC system consisted of a chilled autosampler (Model AS-950, Jasco, Great Dunmow, UK), a single pump (Model 980-PU, Jasco), a degassing unit (Model DG 980-50, Jasco), a low-pressure mixing unit (Model LG 980-02, Jasco) and a variable-wavelength UV detector (975-UV, Jasco). A VG MultiChrom Data Acquisition System (VG Data Systems, Altringham, UK) was used for calculation of results. Samples were centrifuged in a Heraeus Micro-centrifuge (Biofuge A).

2.3. HPLC conditions

The chromatographic system consisted of a polymeric reversed-phase PLRP-S HPLC column (150× 4.6 mm I.D., 5 μm; Polymer Labs., Church Stretton, UK) with an in-line 2-μm pre-filter. Chromatographic analyses were performed at ambient temperature with a mobile phase of 10.5% (v/v) acetonitrile in 20 m*M* ammonium dihydrogen orthophosphate (pH 2.75) at a flow-rate of 1 ml/min. The UV detection wavelength used was 260 nm and sample aliquots of 100 μl were injected into the chromatographic system.

2.4. Preparation of standard solutions

Duplicate stock standard solutions were prepared. Approximately 55 mg of cephradine hydrate (6%, w/w, water) was accurately weighed and dissolved in 50 ml of deionised water to give a nominal concentration of approximately 1 mg/ml cephradine. Dilutions of this were made with deionised water to give working solutions of approximately 500, 100, 50, 10 and 5 μg/ml, respectively. The cephaloridine internal standard solution was prepared by accurately weighing approximately 10 mg of cephaloridine and dissolving it in 10 ml of deionised water to give a nominal concentration of approximately 1 mg/ml. A 15 μg/ml working solution was prepared by taking 300 μl of 1 mg/ml cephaloridine solution and making it up to 20 ml with 4% (v/v) perchloric acid. Stock solutions were stored at +4°C in darkness to minimise photo degradation. The primary stocks were stable for 1 month at this temperature. The

working solutions for cephradine and cephaloridine were prepared on a daily basis.

2.5. Preparation of calibration standards

Calibration standards (0.20, 0.50, 2.01, 4.97, 10.06, 20.13 and 29.60 μg/ml) were prepared by diluting appropriate volumes of cephradine stock solutions to a 1 ml volume, using blank human plasma. Calibration standards were prepared on a daily basis.

2.6. Validation control samples

Validation control samples were prepared to determine the precision and accuracy of the method, and to evaluate the stability of cephradine in human plasma samples. Validation control samples at approximately 0.2, 5, 15 and 30 μg/ml were prepared by diluting appropriate volumes of cephradine stock solutions to a 4 ml volume, using blank human plasma. All validation control samples were aliquoted (200 μl) into 1.5-ml polypropylene microcentrifuge tubes and stored at nominally −20°C.

2.7. Preparation of spiked plasma samples

Calibration standards, validation control samples, or plasma unknowns were prepared by aliquoting 200 μl into 1.5-ml microcentrifuge tubes followed by the addition of 200 μl of internal standard (15 μg/ml cephaloridine in 4%, v/v, perchloric acid). For plasma blank samples, 200 μl of 4% (v/v) perchloric acid was added to 200 μl of drug-free plasma. All samples were mixed by vortex agitation for 5 s and centrifuged for 5 min at 1500 *g*. Portions of the supernatant were transferred to autosampler vials and 100 μl injected into the HPLC system.

2.8. Method validation

During the validation the following parameters were assessed; purity and identity of test compound, verification of calibration model, specificity, limits of quantification, accuracy and precision and compound stability in analytical stock solutions, processed sample extracts, biological matrix and during freeze–thaw cycles [11].

2.8.1. Purity and identity of test compounds

Purity and identity information were provided on the certificates of analysis of the test compound cephradine and the internal standard cephaloridine. Additionally HPLC chromatograms were visually examined for the presence of related peaks.

2.8.2. Verification of calibration model

The suitability of a linear calibration model with weighting factor 1/concentration for cephradine was verified by visual examination of the calibration curves produced on each validation run. This verification is required to ensure that the chosen model adequately describes the relationship between response function (y) and concentration (x). The difference between the observed y-values and the fitted y-value or residual, were checked from each of the validation runs. The most common occurrence is an increase in variance with increase in concentration and this is best managed with a reciprocal power of concentration as the weighting factor.

2.8.3. Specificity

Control blank human plasmas from six different sources were analysed during the validation. In addition, pre-dose clinical study samples were analysed. In all cases chromatograms were visually examined for potential interfering peaks.

2.8.4. Limits of quantification

The lower and upper limits of quantification (LLOQs and ULOQs, respectively) were established by examination of the accuracy and precision data. These limits were defined as the analyte concentration which could be measured in validation control samples with an accuracy not exceeding ±15%, together with a precision not exceeding 15%.

2.8.5. Accuracy

The accuracy of the method (% bias) was determined by assessing the agreement between the measured and nominal concentrations of validation control samples (see below), the measured concentration being the mean of the concentrations obtained

during the precision assessment (see Section 2.8.6), in which four validation control samples at 0.2, 5, 15 and 30 μg/ml were analysed in replicates of six, on four separate occasions.

2.8.6. Precision

The precision of the method (RSD, %) was determined by assessing the agreement between replicate measurements of validation control samples. Validation control samples at 0.2, 5, 15 and 30 μg/ml were analysed in replicates of six, on four separate occasions. The data collated for cephradine were analysed by a one-way analysis of variance (ANOVA) using an ANOVA macro within Excel (Microsoft) to give estimates of the inter- and intra-assay precision of the method [12].

2.8.7. Stability

2.8.7.1. Stability of analytical standard solutions. The stability of analytical standard solutions of cephradine at one concentration (in replicates of six), stored at +4°C for 45 days, was assessed by the comparison of the mean peak areas against those of freshly prepared solutions within the same assay. The percentage difference and the 90% confidence interval for this difference were calculated using natural logarithmic transformed data. Differences were assessed on the basis of their statistical significance and their relevance. If the confidence interval includes zero there is no statistical difference. The difference is not considered relevant if the lower limit of the confidence interval is greater than −10% and the upper limit of the confidence interval is less than +10%.

2.8.7.2. Stability of processed sample extracts. The stability of cephradine in processed extracts of spiked calibration samples stored at +4°C was assessed. The peak area ratios of processed sample extracts stored for 29 h were compared with those of freshly prepared processed sample extracts.

2.8.7.3. Stability in biological matrix. Spiked control samples at three concentrations (0.30, 5.02 and 15.02 μg/ml) were prepared in bulk, aliquoted (200 μl) and then stored at −20°C for 35 days. Spiked control samples at the same three concentrations were prepared fresh on the day of analysis and the stored control samples were allowed to thaw and analysed (n=4) in the same run. The mean determined concentrations at each level and the % differences between stored and fresh spiked control samples were calculated. Stability was assessed as in Section 2.8.7.1.

2.8.7.4. Stability during freeze–thaw cycles. Spiked control samples at three concentrations (0.30, 5.02 and 15.02 μg/ml) were prepared in bulk, aliquoted (200 μl) and then stored at −20°C. Once the samples were fully frozen four aliquots at each concentration were removed from the freezer and allowed to thaw, the aliquots were then returned to the freezer. This procedure was carried out on a further two occasions and all the samples assayed in the same analytical batch alongside freshly prepared spiked control samples. The mean determined concentrations at each level and the % difference between stored and fresh spiked control samples were calculated. Stability was assessed as in Section 2.8.7.1.

3. Results and discussion

3.1. Verification of calibration model

Visual examination of the calibration curves indicated the linear regression model weighted 1/concentration was a suitable model (n=18). The calibration relationship was non-linear in the absence of such a weighting. A typical calibration curve for cephradine over the concentration range 0.2 μg/ml to 30 μg/ml is presented in Fig. 1.

3.2. Specificity

Examination of HPLC chromatograms of analytical stock solutions of cephradine and cephaloridine showed a known synthetic impurity in the cephradine chromatograms, that of cephalexin, at not more than 5% (w/w) in the cephradine standard. This cephalexin peak had a retention time at 8.5 min and was well resolved from cephradine and cephaloridine (Fig. 4). Visual inspection of HPLC chromatograms obtained from extracted blank and spiked human plasma from

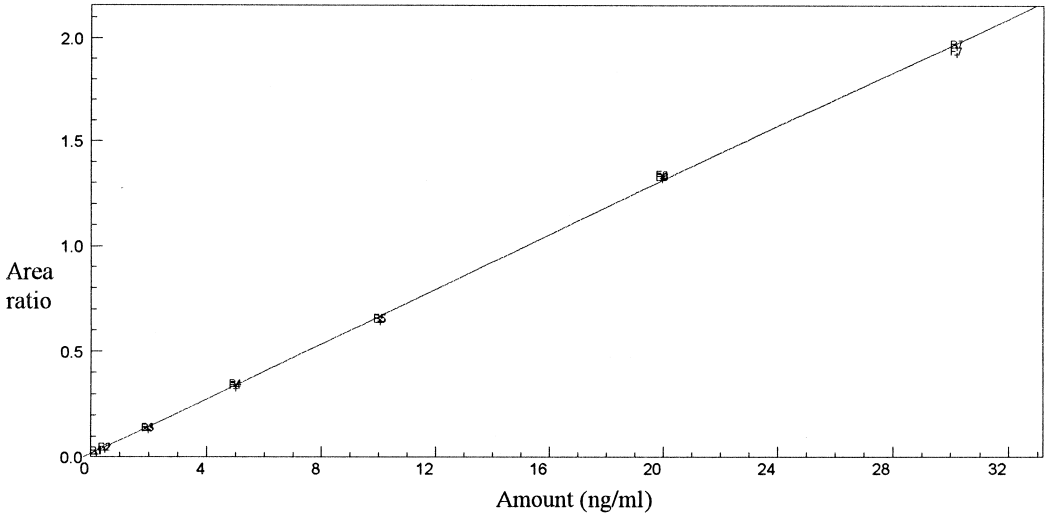

Fig. 1. Typical calibration curve for cephradine in human plasma over the range 0.2 to 30 μg/ml.

six different sources revealed a peak at 7.8 min which was well resolved from the peaks of interest (Figs. 2–4). No other significant peaks were noted in the chromatograms.

Approximate retention times for cephalexin, cephradine and cephaloridine were 8.5, 10 and 12 min, respectively. Fig. 2 depicts a typical chromatogram of an extracted blank human plasma sample.

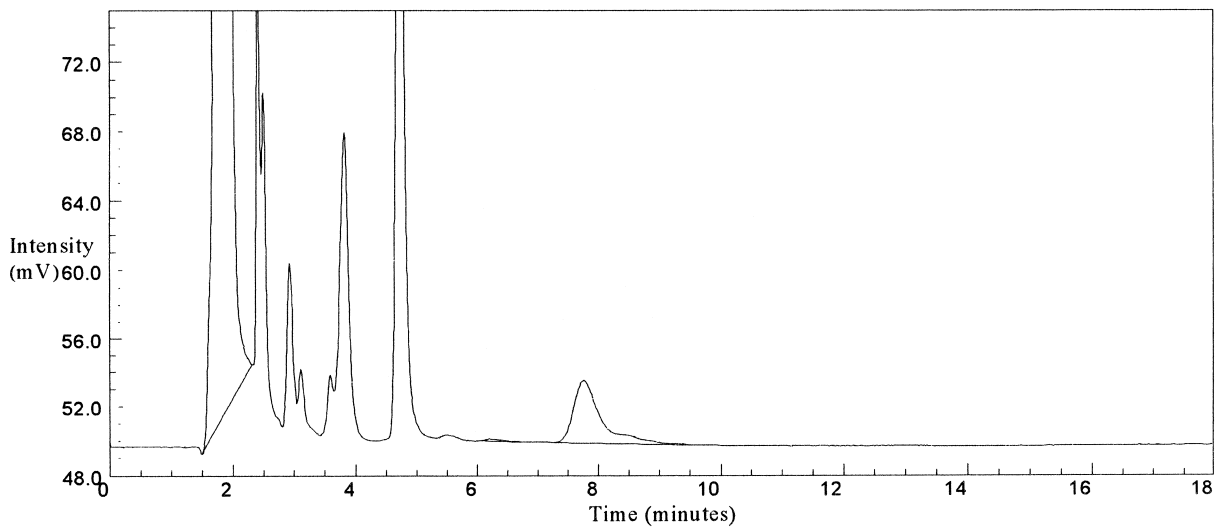

Fig. 2. Typical chromatogram of a blank human plasma sample.

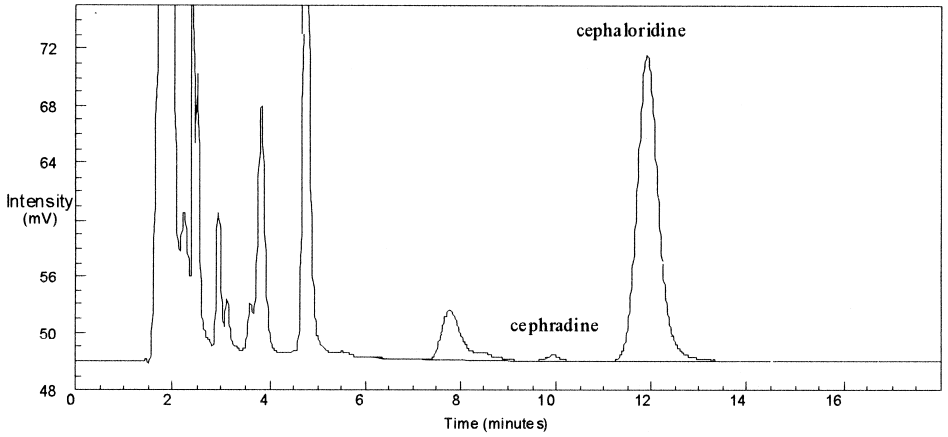

Fig. 3. Typical chromatogram of cephradine standard, 0.205 μg/ml (LLOQ).

3.3. Limits of quantification

As defined by the lower and upper validation control concentrations possessing acceptable accuracy and precision (see Sections 2.8.5 and 2.8.6, respectively), the limits of quantification of the method were 0.2 and 30 μg/ml. The accuracy of the method at 0.2 μg/ml was −4.1% with a precision at 2 μg/ml of 4.9% (intra-assay) and negligible (inter-assay), respectively. The accuracy at 30 μg/ml was −5.4% with a precision at 30 μg/ml of 3.5% (intra-assay) and 3.9% (inter-assay), respectively. Figs. 3 and 4 depict representative chromatograms of cephradine spiked at 0.205 and 30.18 μg/ml in human plasma, respectively.

3.4. Accuracy

The accuracy (% bias) of the method for cephradine is summarised in Table 1. At all spiked validation control concentrations of cephradine examined, the bias was less than ±15% and, therefore, acceptable. The maximum % bias was 7.2.

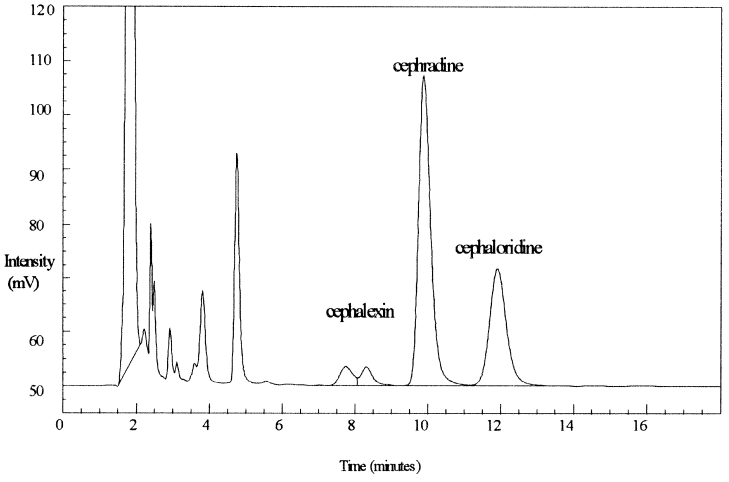

Fig. 4. Typical chromatogram of cephradine standard, 30.18 μg/ml (ULOQ).

Table 1
Accuracy and precision of the method for cephradine (n=18)

Cephradine concentration (μg/ml)	Accuracy (% bias)	Intra-assay precision (RSD, %)	Inter-assay precision (RSD, %)
0.2	−4.1	4.9	Negligible
5.0	−7.2	2.7	1.5
15.0	−3.6	4.8	2.2
30.0	−5.4	3.5	3.9

3.5. Precision

Estimates of the inter- and intra-assay precision (RSD, %) of the method for cephradine are summarised in Table 1. At all spiked validation control concentrations of cephradine examined, the inter- and intra-assay precision was less than 15% and, therefore, acceptable. The maximum RSD was 4.9%.

3.6. Stability

3.6.1. Stability of analytical standard solutions

The mean difference between analytical standard solutions (n=6) stored at +4°C for 45 days and freshly prepared standards was −2.4%. The 90% confidence interval of this difference (−1.2 to −3.5%) did not include zero, indicating a significant difference. However, the difference was not considered relevant as the lower and upper limits of the confidence interval were within ±10%.

It was, therefore, concluded that cephradine analytical standard solutions are stable when stored at +4°C for at least 45 days. In practice, new analytical stock solutions were prepared on a monthly basis.

3.6.2. Stability of processed sample extracts

The mean % difference between extracts stored at +4°C on the autosampler rack for 29 h and freshly prepared extracts was −2.99%. This indicated a small amount of degradation, however, individual % differences were both positive and negative and the extracts are considered stable at +4°C for at least 29 h. The stability of cephradine in processed sample extracts is detailed in Table 2.

3.6.3. Stability in biological matrix

The mean difference between spiked plasma samples stored at −20°C for 35 days (Table 3) and freshly prepared samples at 0.30, 5.02 and 15.02 μg/ml was −2.4%, +3.4% and +4.9%, respectively. There was no significant difference at 0.30 and 5.02 μg/ml as the lower limits of the confidence interval were greater than −10% and the upper limits of the confidence limit were less than +10%. At 15.02 μg/ml the upper confidence limit was +10.5% indicating a possible significant difference; as this was a positive difference it did not indicate any instability. It was concluded that cephradine is stable in human plasma when stored at −20°C for at least 35 days. The stability of cephradine in human plasma frozen at −20°C is detailed in Table 3.

3.6.4. Stability during freeze–thaw cycles

Cephradine showed no significant difference at 0.30, or 15.02 μg/ml as all three confidence intervals included zero and the lower limits of the confidence interval are greater than −10% and the upper limits of the confidence interval are less than +10%. At 5.02 μg/ml there was a statistically significant difference between data obtained from all

Table 2
Processed sample stability of cephradine in human plasma at +4°C for 29 h

Concentration (μg/ml)	Stored PARs[a]	Fresh PARs[a]	% Difference
0.20	0.0157	0.0140	−10.90
0.50	0.0398	0.0376	−5.50
2.01	0.1497	0.1520	1.50
4.97	0.3631	0.3741	3.00
10.06	0.7401	0.6700	−9.50
20.13	1.5034	1.5452	2.80
29.60	2.1615	2.1126	−2.30
Mean % difference			−2.99

[a] PARs=Peak area ratios.

Table 3
Stability of cephradine in human plasma at −20°C for 35 days

	0.30 μg/ml		5.02 μg/ml		15.02 μg/ml	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Mean	−1.169	−1.194	1.49	1.52	2.59	2.64
Geometric mean	0.31	0.30	4.44	4.59	13.32	13.96
Number	4	4	4	4	4	4
Difference (%)	−2.4		3.4		4.9	
CL (lower limit)	−6.4		−2		−0.5	
CL (upper limit)	1.7		9.2		10.5	

the freeze–thaw cycles, as the confidence interval did not include zero, however, the difference was not considered relevant as the lower limit of the confidence interval was greater than −10% and the upper limit of the confidence interval was less than +10%. It was concluded that cephradine is stable in human plasma when subjected to up to three freeze–thaw cycles.

The stability of cephradine in frozen biological matrix after being subjected to one, two and three freeze–thaw cycles is detailed in Tables 4–6, respectively.

3.7. Application to clinical trial pharmacokinetics

This method was developed in order to provide bioanalytical support to a bioequivalence study in human volunteers. Over 500 plasma samples were analysed in 18 assays, all of which passed the acceptance criteria for calibration standards and quality control samples [11]. The RSD for the gradient on the calibration curves was 7% and the highest RSD and % bias for the quality control samples was 9.2% and −4.1%, respectively. Overall, the method was found to be simple, efficient and

Table 4
Freeze–thaw stability of cephradine in human plasma after one cycle

	0.30 μg/ml		5.02 μg/ml		15.02 μg/ml	
	Fresh	Cycle 1	Fresh	Cycle 1	Fresh	Cycle 1
Mean	−1.187	−1.150	1.61	1.55	2.64	2.62
Geometric mean	0.305	0.317	4.98	4.72	13.98	13.69
Number	4	4	4	4	4	4
Difference (%)	3.8		−5.1		−2.1	
CL (lower limit)	−1.8		−8.1		−5.7	
CL (upper limit)	9.6		−2.0		1.7	

Table 5
Freeze–thaw stability of cephradine in human plasma after two cycles

	0.30 μg/ml		5.02 μg/ml		15.02 μg/ml	
	Fresh	Cycle 2	Fresh	Cycle 2	Fresh	Cycle 2
Mean	−1.187	−1.159	1.61	1.55	2.64	2.61
Geometric mean	0.305	0.314	4.98	4.73	13.98	13.63
Number	4	4	4	4	4	4
Difference (%)	2.8		−4.9		−2.5	
CL (lower limit)	−2.1		−7.2		−6.6	
CL (upper limit)	8.0		−2.6		1.7	

Table 6
Freeze–thaw stability of cephradine in human plasma after three cycles

	0.30 μg/ml		5.02 μg/ml		15.02 μg/ml	
	Fresh	Cycle 3	Fresh	Cycle 3	Fresh	Cycle 3
Mean	−1.187	−1.16	1.61	1.55	2.64	2.62
Geometric mean	0.305	0.313	4.98	4.70	13.98	13.71
Number	4	4	4	4	4	4
Difference (%)	2.7		−5.6		−1.9	
CL (lower limit)	−1.8		−7.2		−5.4	
CL (upper limit)	7.5		−3.9		1.7	

robust. Fig. 5 shows the median semi-log plasma concentration–time profile of cephradine following the oral administration of 500 mg tablets of Kefdrin and Velosef, respectively. The calculated pharmacokinetic data shows the two formulations of cephradine to be bioequivalent. Thus, the $AUC_{0-\infty}$ geometric mean ratio was 1.02 with an associated 90% confidence interval of 0.96 to 1.08 and this interval is well within the generally accepted bioequivalence interval of 0.80 to 1.25. Other pharmacokinetic parameters including AUC_{0-t}, C_{max}, and $t_{1/2}$ also confirmed bioequivalence by similar criteria. The mean area under the curve (AUC) extrapolated for Kefdrin was 2.5% (range 1.4 to 4.7%) and for Velosef was 2.4% (range 1.6 to 3.3%). Therefore, for all subjects, the extrapolated AUC did not exceed 5% of the total $AUC_{0-\infty}$ indicating that the LLOQs of the method are appropriate for the bioanalytical support of this type of bioequivalence study.

4. Discussion and conclusions

We have developed and validated a simple and sensitive HPLC–UV method for the quantification of cephradine in human plasma, which is precise, accurate and has a suitable dynamic range for clinical trial support. The poly(styrene–divinylbenzene) HPLC column was found to be very reproduc-

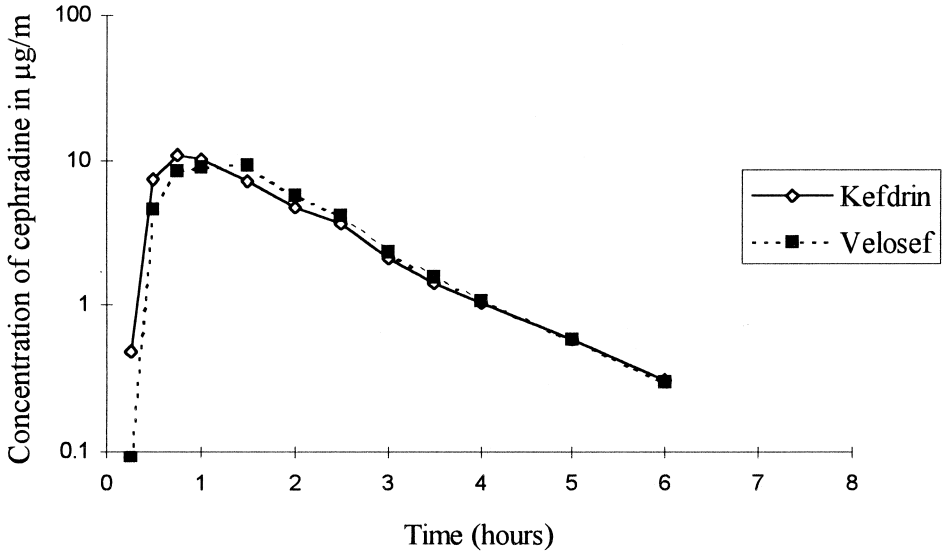

Fig. 5. Median cephradine plasma concentration–time semi-log profile after oral administration of Kefdrin (500 mg) and Velosef (500 mg) to 18 subjects.

ible, with no degradation in column performance as monitored by calibration and quality control statistics over 18 analytical batches. This simple protein precipitation method would easily lend itself to automation if so required in the future. The easiest and cheapest part of the process to automate would be the liquid handling steps with the use of a robotic sample processor (RSP) such as a Packard MultiPROBE. The RSP could easily be programmed to pick up plasma samples followed by acidified internal standard and dispense these into tubes or a 96-well microtitre plate. Following centrifugation (off-line) the RSP could then aspirate the supernatant into a microtitre plate ready for analysis by HPLC–UV. The rate-limiting step of this process if using tubes would be the capacity of the centrifuge. However if a 96-well microtitre plate/s were used with an appropriate centrifuge such as a Heraeus Minifuge T, a total of 384 samples could be prepared simultaneously with each plate being prepared (excluding centrifugation time) in approximately 35–40 min. Full automation of the method would prove more costly and difficult as an on-line centrifuge would be required along with an automation partner such as a Zymate XP robot to act as an interface between the two. A third option for automation currently under development in our laboratories [13] involves the use of automated protein precipitation by filtration in the 96-well format thereby removing the need for centrifugation.

The protein precipitation HPLC–UV method for plasma cephradine has been shown to be accurate, precise and of appropriate sensitivity and selectivity for its successful application to a strictly controlled bioequivalence study with 500 mg oral doses of Kefdrin and Velosef in human volunteers. We believe that this method is suitable for transfer to other laboratories with simple HPLC–UV equipment at their disposal. Future work will include an investigation on the validity of the method to more general clinical situations where concomitant medication is being prescribed.

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