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Quantitation of efletirizine in human plasma and urine using automated solid-phase extraction and column-switching high-performance liquid chromatography

R.A. Coe[a], L.S. DeCesare[b], J.W. Lee[a,*]

[a]*MDS Harris, Inc., 621 Rose Street, Lincoln, NE 68501, USA*
[b]*UCB Pharma, Inc., Smyrna, GA 30080, USA*

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Abstract

A heart-cut column-switching, ion-pair, reversed-phase HPLC system was used for the quantitation of efletirizine (EFZ) in biological fluids. The analyte and an internal standard (I.S.) were extracted from human EDTA plasma by C_{18} solid-phase extraction (SPE) using a RapidTrace® workstation. The eluent from the SPE was evaporated, reconstituted and injected onto the HPLC column. Urine samples were diluted and injected directly without the need of extraction. The compounds of interest were separated from most of the extraneous matrix materials by the first C_{18} column, and switched onto a second C_{18} column for further separation using a mobile phase of stronger eluting capability. Linearity range was 10–2000 ng ml^{-1} for plasma and 0.05–10 μg ml^{-1} for urine. The lower limit of quantitation (LOQ) was 10 ng from 1 ml of plasma, with a signal-to-noise ratio of 15:1. Inter-day precision and bias of quality control samples (QCs) were <5% for plasma and <7% for urine. Selectivity was established against six other antihistamines, three analogs of efletirizine, and on 12 control plasma lots and nine control urine lots. Recovery was 90.0% for EFZ and 89.5% for I.S. from plasma. One hundred samples can be processed in every 2.75 h on a 10-module RapidTrace® workstation with minimal human attention. Method ruggedness were tested on three brands of SPE and six different lots of one SPE brand. Performance ruggedness was demonstrated by different analysts on multiple HPLC systems. Analyte stability through sample storage, extraction process (benchtop, freeze–thaw, refrigeration after extraction) and chromatography (on-system, reinjection) was established.

Keywords: Efletirizine

1. Introduction

Efletirizine (EFZ), {2-[4-[bis(4-fluorophenyl)-methyl]-1-piperazinyl]ethoxy]acetic acid} (Fig. 1a), is an antihistamine under investigation. It has potent antihistaminic activity because of its selectivity and high affinity with the H_1-receptors [1]. The in vivo action is rapid, potent, selective and long-lasting at both pulmonary and cutaneous sites [2]. No extensive metabolism was observed in man. In order to support the clinical trials, a reliable, sensitive and selective bioanalytical method was required. So far, no analytical method has been reported in the literature for EFZ in biological fluids.

Bioanalytical methods of a similar antihistamine,

*Corresponding author. Fax: +1-402-476-7598.
E-mail address: jwl@mdsharris.com (J.W. Lee)

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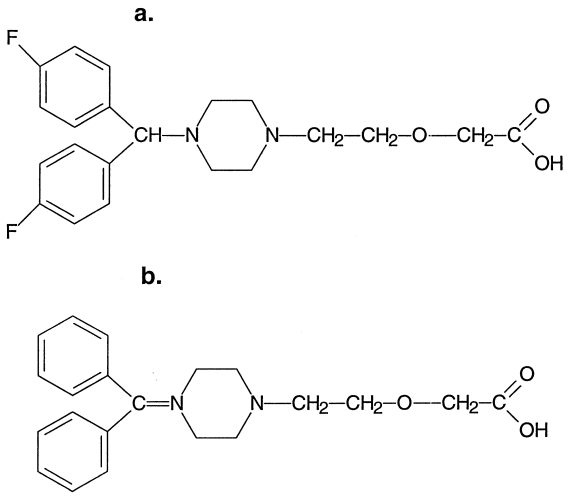

Fig. 1. Structures of EFZ (a) and I.S. (b).

cetirizine, have been reported [3–8]. Protein precipitation and liquid–liquid extraction with an organic solvent were used in these methods. The analyte was separated and quantitated by isocratic high-performance liquid chromatography (HPLC) with UV detection [3–5] or diode array detection (DAD) [6], gas chromatography–nitrogen–phosphorus detection (GC–NPD) [7], or high-performance thin-layer chromatography (HPTLC) [8].

It is important to develop and validate a reliable and reproducible method that can sustain the vigorous test of time through months of clinical studies in a drug program. An automated workstation such as RapidTrace® can be used to set up multitudes of programs to test various solid-phase extraction (SPE) sorbents and method protocols to speed up development and optimization processes. Once the method is finalized, all validation and sample analysis are run by the same program. Therefore, SPE automation would minimize human variability during sample analysis. Automated extraction equipment for SPE has been used in the laboratory to improve extraction consistency and reduce the labor of sample processing [9].

This paper describes the method development and validation of an HPLC method for the quantitation of EFZ in human plasma and urine.

2. Experimental

2.1. Instrumentation

A two-dimensional HPLC system was used. The first analytical column was a Primesphere C_{18}, 150× 4.6 mm, 5 μm from Phenomenex (Torrence, CA, USA). The second column was a Spherisorb ODS2, 150×4.6 mm, S3 (3 μm), from Waters (Milford, MA, USA). Column temperature was maintained at 40°C by a Eppendorf Model CH-30 column heater and controller Model TC-50 (Madison, WI, USA). Mobile phases were: (A) acetonitrile–potassium phosphate (0.01 *M*, pH 3.0)–10 $mmol^{-1}$ 1-octanesulfonic acid (42:58, v/v) for the first column, and (B) acetonitrile–potassium phosphate (0.01 *M*, pH 3.0)–10 $mmol^{-1}$ 1-octanesulfonic acid (48.5:51.5, v/v) for the second column. A Valco VICI 10-port valve-switcher (Houston, TX, USA) was plumbed in the configuration described in Fig. 2. The HPLC system was comprised of a WISP 712 autosampler from Waters, and two pumps of either Waters 510 or 501. Flow-rate was 1.0 ml min^{-1}. Run time was 17 min. The eluate was monitored at 222 nm with a Thermo Separations LDC 3200 detector (Riviera Beach, FL, USA). Injection volume was 80 μl and 100 μl for plasma and urine samples, respectively.

A second HPLC system used a Model 484 detector from Waters, with other components identical to the first system. Analytical columns from different batches of packing material were tested between the two systems and the chromatographic performances were similar.

2.2. Materials

ucb 28754 (efletirizine dihydrochloride), internal standard (I.S.) (ucb 20028, Fig. 1b), ucb 29429, ucb 29496, ucb 30287, cetirizine hydrochloride and pseudoephedrine were supplied by UCB Pharma (Smyrna, GA, USA). Terfenidine and hydroxyzine were purchased from USP (Rockville, MD, USA). Norterfenidine was purchased from MDS Isotopes (Pointe-Claire-Dorval, Quebec, Canada). Astemizole was purchased from Janssen Research Products (Flanders, NJ, USA), and desmethylastemizole was

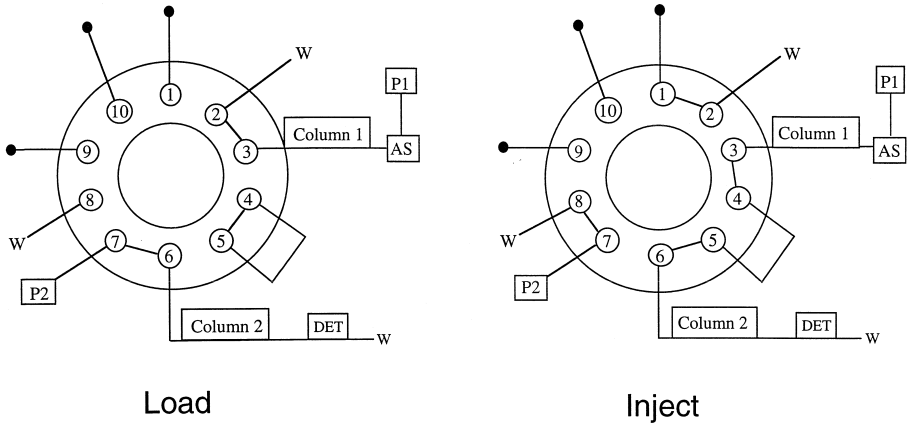

Fig. 2. Configuration of column switch valve. W=Waste, P1=pump 1, P2=pump 2, AS=autosampler, DET=detector, (·)=port closed.

purchased from High Standard Products (Inglewood, CA, USA). All chemicals used were of analytical or HPLC grade. The organic solvents were from Fisher (Fair Lawn, NJ, USA) with the exception of 1-octanesulfonic acid which was purchased from Sigma (St. Louis, MO, USA). Inorganic chemicals were from Mallinckrodt (Paris, KY, USA). SPE cartridges were Bond-Elute C_{18}, 200 mg/3 ml from Varian (Harbor City, CA, USA). Mobile phases were vacuum-filtered through 0.45-μm nylon membranes from Gelman Sciences (Ann Arbor, MI, USA). Deionized water was prepared in the laboratory using a NANOpure water purification system from Barnstead (Dubuque, IA, USA). Control human ethylenediaminetetraacetic acid (EDTA), citrated, Na^+- and Li^+-heparinized plasma were purchased from Nashville Biological (Cincinnati, OH, USA).

2.3. Preparation of solutions

Primary stock solutions for standards and QCs were prepared from separate weighings. The primary stock solutions of EFZ at approximately 1 mg ml^{-1} in water were diluted to form a series of nine spiking standards at concentrations of 0.01–20 μg ml^{-1}. These solutions were stored at 2–8°C in polypropylene tubes for at least one month. Plasma working standards were prepared daily by adding 100 μl of the spiking standards to 1.0 ml of control plasma. The standard concentrations were 10, 20, 50, 100, 500, 1000, 1300, 1700 and 2000 ng ml^{-1}. QCs were prepared in volumetric flasks, polypropylene tubes and stored at −20°C with the clinical samples until assayed. Another group of QCs was set aside and stored separately at −20°C for long-term storage stability tests.

Working standards were diluted in urine at the concentrations of 0.05, 0.10, 0.50, 1.0, 2.0, 5.0, 8.0 and 10 μg ml^{-1}. These standards were stored at 2–8°C in polypropylene tubes for no more than seven days.

2.4. Sample processing procedures

2.4.1. Plasma

A 1-ml aliquot of QC or analytical sample was added to a 100×13 mm polypropylene culture tube. After adding 100 μl of I.S. (6 μg ml^{-1} in water) and 1.0 ml of phosphate buffer (0.05 *M*, pH 5.30) and mixing, all samples were centrifuged for 5 min. and transferred to clean tubes. The samples were loaded onto the RapidTrace® workstation from Zymark (Hopkinton, MA, USA). The automated SPE procedure was programmed to performed the following: conditioning the cartridges with 2 ml each of methanol, water and potassium phosphate buffer (0.05 *M*, pH 6), loading the buffered plasma sample, washing the cartridges with 6 ml of water followed by 2 ml of methanol–water (40:60, v/v), then drying for 2 min with N_2. The analytes were eluted with 2 ml of

56.5% ammonium hydroxide–methanol (2:98, v/v), and evaporated to dryness under N_2 at 40°C in a Zymark Turbovap LS®. The samples were reconstituted with 200 μl water and the extracts were transferred into an autosampler vial with plastic inserts.

2.4.2. Urine

A 200-μl aliquot of working standard, QC or analytical sample was added to a glass autosampler vial. After adding 200 μl of I.S. and 600 μl of potassium phosphate buffer (0.01 *M*, pH 3), the samples were capped and mixed by inversion.

2.5. Data regression

Chromatograms were measured using a VG® Multichrom data system for VAX®/VMS. The raw data output was acquired on a VG® Chromserver and then transferred to the VAX®/VMS. Response in peak height ratio of each analyte to the I.S. (y) was plotted against analyte concentration (x). A linear regression with $1/x$ weighting was used to determine slopes, intercepts and correlation coefficients. Unknowns were calculated by the equation: $x=[y-(y-\text{intercept})]/\text{slope}$.

Usually, multiple replicates of samples (n) are extracted to generate statistical mean and relative standard deviation (RSD) values.

3. Results and discussion

3.1. Method development

3.1.1. Plasma

The first HPLC set-up was an isocratic system with a single C_8 column and mobile phase of acetonitrile–potassium phosphate buffer (0.01 *M*, pH 3) (45:55, v/v).

The functional groups of EFZ have pK_a values of approximately 1.67, 2.95 and 8.27. These functional groups will assume a positive or negative charge at most pH conditions, increasing the polarity of the EFZ molecule. Although there are hydrophobic functional groups, the charged molecule may not be effectively extracted with an organic solvent. Analytical methods reported on cetirizine, an antihistamine with similar functional groups, used protein precipitation [3] or liquid–liquid extraction with polar organic solvents [5].

We tried liquid–liquid extraction followed by adsorption on diatomaceous earth and elution with ethyl acetate. Another procedure of adjusting plasma samples to pH 5 and extraction with ethyl acetate was also attempted. Poor, inconsistent recovery and noisy baseline in the HPLC chromatography were observed with these methods. Samples precipitated with perchloric acid also contained multiple matrix interferences. SPE sample processing was desirable because interferences can be removed through vigorous washings once the compounds of interest are adsorbed onto the sorbent. Moreover, SPE is adaptable to automation.

Since the two tertiary amines on the piperazine function group are charged at pH 6, ion-exchange SPE was attempted first. The plasma was adjusted to pH 6 before loading onto the SPE column. After washing with water, the compounds of interest were eluted with methanol. Recovery was poor even when the columns were eluted with a strong basic solvent. A selection of reversed-phase SPE columns were screened, including C_2, C_8 and C_{18}, and from several different vendors. Recovery of both EFZ and I.S. were >95% on all of the aliphatic chain bonded phases. However, the target limit of quantitation (LOQ) was not quantifiable because of interfering matrix peaks and late-eluting sample components.

C_2 and C_{18} SPE columns were selected for further tests with varying strength of organic solvent washes. C_2 may not retain some of the interfering material, while C_{18} may retain the analytes strongly to allow removal of the extraneous materials by more vigorous washings and/or stronger solvents. After sample loading, 1 ml wash solvents of 0–50% methanol or acetonitrile in pH 6 phosphate buffer were tested. The RapidTrace® workstation SPE processing units were programmed to prepare wash solutions in the mixing chamber with increments from 20 to 50% methanol (or acetonitrile) in buffer. The RapidTrace® then applied the washes to the appropriate set of test samples. Thus, a single experiment could be carried out without the manual labor of preparing eight separate solutions.

Recovery results were similar between the methanol and acetonitrile solvents – when wash solvent

strength was >40%, recovery decreased to <80%. From 0 to 40% wash solvent, recovery of the analytes was at least 90%. However, interference at the analyte retention time and late-eluting peaks were observed. Washes with buffers at pH 3–6 did not improve the chromatography. In addition, standard linearity was not achieved because the recovery of low concentration samples was lower than that of the high concentration. Since there was possible secondary ion-exchange interaction between the analyte amine group and the SPE sorbent, elution solvents varied in pH and ion-pairing reagent were tested for elution. The results are summarized in Table 1. Recoveries were inconsistent when eluting solvents of methanol without and with triethylamine (TEA) or HCl were used. The addition of 1-octanesulfonic acid resulted in better consistency in recovery. Acidic methanol produced large matrix interferences under EFZ. With ammonium hydroxide–methanol (2:98) as the eluting solvent, linearity was improved and the recovery at the LOQ was higher than that of 1-octanesulfonic acid–methanol solvent. The RSDs in Table 1 of EFZ recovery showed that the recovery was consistent for the low, middle and high concentrations of the standard calibration range using the ammonia–methanol elution solvent.

HPLC with heart-cut column-switching was applied to resolve EFZ and I.S. from the matrix components. The compounds of interest were separated from endogenous matrix peaks by the highly endcapped, Primesphere column and eluted onto a second column. The Spherisorb ODS2 column provided the second dimensional separation. Fig. 2 shows the configuration of the column switch valve. Selectivity was best achieved when the heart-cut fraction was made as small as possible. The solvent concentration of the first mobile phase was adjusted to retain the compounds of interest such that no matrix peaks interference occurred in the final chromatography. It was found that a retention-time window of approximately 6.5–7.0 min on column 1 gave the cleanest chromatography for EFZ and I.S., without an excessive injection cycle-time (Figs. 3 and 4). The solvent strength of mobile phase 2 was made stronger than mobile phase 1 to prevent band-broadening. The acetonitrile concentration of mobile phase 2 was then adjusted to keep the run time at less than 20 min.

3.1.2. Urine

Urine samples did not need to be extracted since the expected concentrations of the clinical samples would be at least five times higher than the plasma samples. The same heart-cut HPLC system described for the plasma gave sufficient selectivity to allow direct injection of the five-fold diluted urine samples without SPE processing.

3.2. Selectivity

Twelve lots of control EDTA plasma and nine lots of control urine were tested. All lots showed no interferences at the retention times of the compounds of interest. Representative chromatograms of the control matrices were shown in trace 2, Fig. 5 for plasma and trace 4, Fig. 6 for urine. Ten other

Table 1
SPE eluent tests and recovery results of EFZ[a]

	10 ng ml^{-1}	100 ng ml^{-1}	2000 ng ml^{-1}	Elution solvent
% Recovery	55.4	71.5	85.9	A
	51.1	64.5	81.3	B
	[b]	[b]	[b]	C
	76.8	93.5	88.4	D
	90.7	89.5	89.9	E
RSD of recovery (%) (n=6)	8.28	1.27	1.28	E

[a] Recovery of EFZ at concentrations of 10, 100 and 2000 ng ml^{-1} was tested with four SPE elution solvents: (A) methanol, (B) 0.05 mol l^{-1} TEA in methanol, (C) 1 mol l^{-1} HCl in methanol, (D) 10 $mmol^{-1}$ 1-octanesulfonic acid in methanol, or (E) 2% ammonium hydroxide in methanol. The spiked plasma standards were compared to the unextracted solutions prepared in an interference-free matrix at the same concentrations.

[b] = Unable to calculate due to large matrix interference.

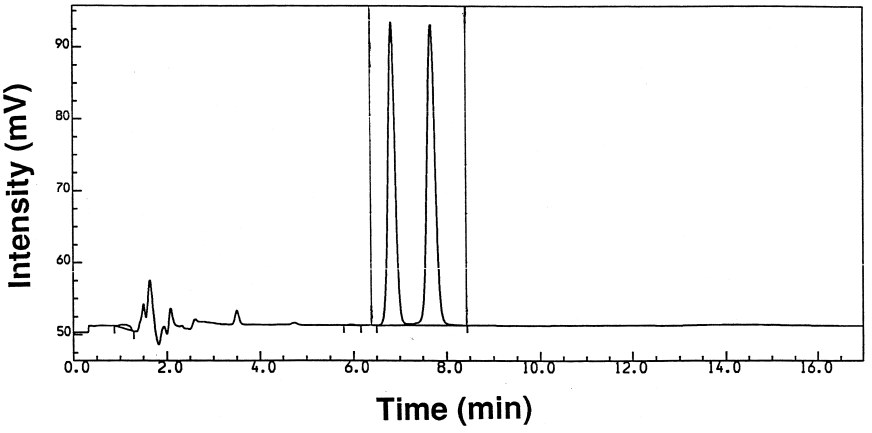

Fig. 3. Compounds of interest injected onto column 1 with valve-switch times chosen.

compounds including two potential metabolites of EFZ, six other antihistamines, and one known metabolite in animal species were tested at concentrations ranging from 400 to 1000 ng ml^{-1}. No interference was observed for all compounds tested: EFZ, I.S. and a known metabolite, ucb 29429, were chromatographically separated with retention times at 13.2, 15.2 and 14.6 min on HPLC system 1, respectively, and at 13.8, 15.8 and 15.1 min on HPLC system 2, respectively. Two potential metabolites, ucb 29496 and ucb 30287, and the other antihistamines were not detected due to either a difference in their UV absorbance from that of EFZ and I.S., or not eluted within the heart-cut window.

The effect of anticoagulants was tested on the quantitation of EFZ. Plasma samples collected into sodium heparin, lithium heparin and sodium citrate all gave similar results to those samples collected with EDTA.

3.3. Recovery

Recovery of EFZ from plasma samples was determined by comparing extracted plasma samples spiked with EFZ and I.S. to neat solution samples of the same concentrations. Extraction recovery of EFZ was 89.9–90.7% over the range 10–2000 ng ml^{-1} (Table 1). Recovery of the internal standard was

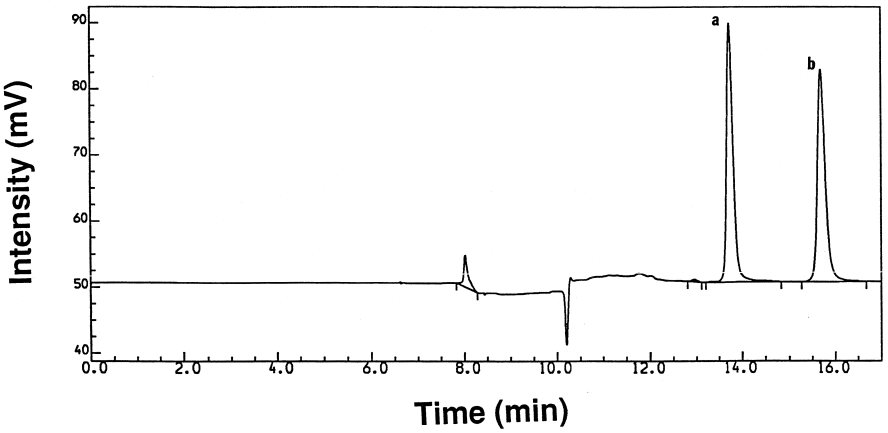

Fig. 4. Compounds of interest eluted from column 2 after final separation. (a) EFZ, (b) I.S.

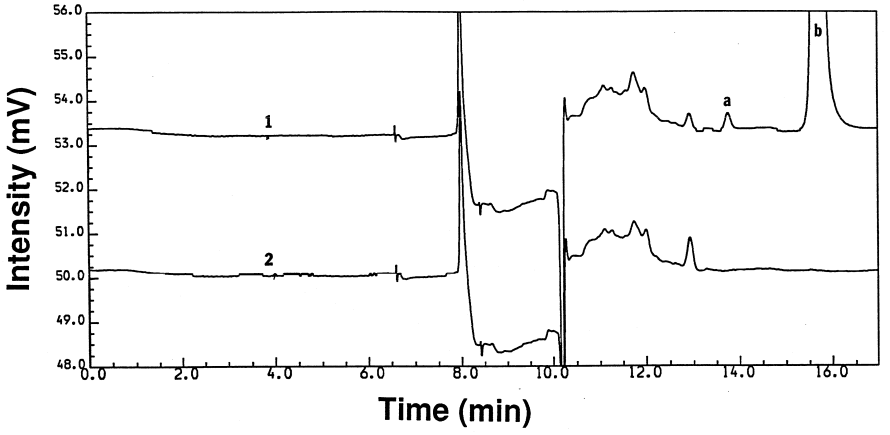

Fig. 5. (1) Extracted LOQ standard 10 ng ml^{-1}, (2) extracted control human EDTA plasma. (a) EFZ, (b) I.S.

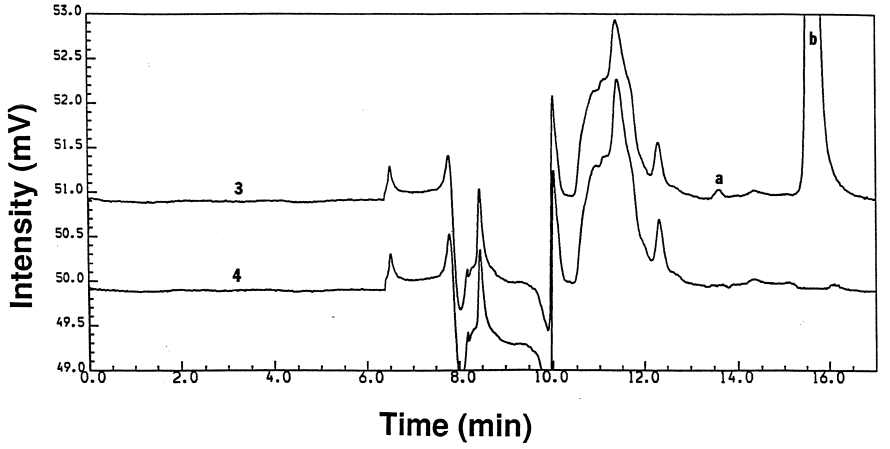

Fig. 6. (1) Extracted LOQ standard 10 ng ml^{-1}, (2) extracted control human urine. (a) EFZ, (b) I.S.

89.5%. Since urine samples were not extracted, recovery should be 100%.

3.4. Linearity, bias and precision

3.4.1. Plasma

The standard curve was linear over the concentration range 10–2000 ng ml^{-1} with a correlation coefficient of $r^2>0.999$, slope of 0.0012, *y*-intercept of 0.0010, and *x*-intercept of −0.7975. Table 2 shows the standard curve summary of five validation runs for plasma samples. They were performed on two HPLC systems and by two analysts over 12

Table 2
Precision and bias of plasma inter-day calibration standards

Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	RSD (%)	RE (%)
10.0	9.64	2.4	−3.6
20.0	20.1	1.5	+0.4
50.0	50.1	1.9	+0.2
100	101	1.0	+1.0
500	512	0.6	+2.3
1000	1010	2.4	+0.7
1300	1300	2.3	+0.2
1700	1680	2.0	−1.2
2000	2000	2.8	−0.1

days. The RSD was ≤2.8% for spiked plasma standards of EFZ. The LOQ at 10 ng ml^{-1} was the concentration that met the pharmaceutical industry's acceptance criteria of <20% relative error (RE) and RSD for bias and precision [10]. The signal-to-noise ratio at this concentration was 15:1, estimated from performance of two HPLC systems. Inter-day precision of the LOQ from five analytical runs was 2.4% RSD, with −3.6% RE (Table 2), intra-day precision of the LOQ was 7.7% RSD, $n=12$, from six different control plasma lots. Fig. 5 shows a typical chromatogram of the LOQ compared to the blank control plasma. Inter-day precision from plasma QCs determined from assaying six replicates each of four concentrations was ≤4.3% RSD (Table 3). The intra-day precision of QCs was 2.6% or less.

3.4.2. Urine

The standard curve for urine samples was linear over the concentration range of 0.05–10.0 μg ml^{-1} with a correlation coefficient of $r^2>0.999$, slope of 0.0034, *y*-intercept of 0.0018, and *x*-intercept of −0.0135. The RSD from five validation analytical runs over six days was ≤4.6% for spiked urine standards of EFZ (Table 4). The LOQ was 0.05 μg ml^{-1}. The signal-to-noise ratio at this concentration was 13:1, estimated from the performance of two HPLC systems. Inter-day precision of the LOQ from five analytical runs was 4.6% RSD. Fig. 6 shows a typical chromatogram of the LOQ compared to the blank control. Inter-day precision from urine QCs determined from assaying six replicates each of low, medium and high concentrations was ≤4.8% RSD (Table 5). The within-run precision of control samples was ≤8.9% RSD.

Table 3
Precision and bias of plasma quality control samples

Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	RSD (%)	RE (%)
Intra-day ($n=6$)			
30.0	31.2	1.8	+3.9
300	320	1.8	+6.7
900	949	1.8	+5.4
1500	1540	2.6	+2.6
Inter-day ($n=30$)			
30.0	31.3	3.7	+4.2
300	314	4.3	+4.7
900	942	3.5	+4.6
1500	1510	2.6	+0.6

Table 4
Precision and bias of urine inter-day calibration standards

Concentration added (μg/ml)	Concentration found (μg/ml)	RSD (%)	RE (%)
0.050	0.0515	4.6	+3.1
0.100	0.101	4.4	+1.2
0.500	0.485	0.8	−2.9
1.00	0.985	0.9	−1.5
2.00	1.99	0.4	−0.4
5.00	4.99	0.4	−0.2
8.00	8.02	0.3	+0.2
10.0	10.0	0.4	+0.2

3.5. Stability

Stability testing of the analytes was performed on plasma samples before and after extraction. When processed under fluorescent lighting, the analytes were stable in the plasma matrix at room temperature for up to 29.5 h, and at −20°C for up to 31 weeks. Urine samples were stable under fluorescent lighting at room temperature for four days, and at −20°C for seven months. There was no effect on the analytical results after three cycles of freezing and thawing of plasma or urine samples. The analytes were stable on the autosampler tray for 59 h for the reconstituted plasma extract and 36 h for the diluted urine, as determined by reinjection. They can also be stored in a refrigerator for at least 90 h before injection.

Table 5
Precision and bias of urine quality control samples

Concentration added (μg/ml)	Concentration found (μg/ml)	RSD (%)	RE (%)
Intra-day ($n=6$)			
0.150	0.147	8.9	−1.8
1.50	1.63	0.8	+8.4
7.50	7.93	0.9	+5.7
Inter-day ($n=30$)			
0.15	0.149	4.8	−0.07
1.5	1.60	1.5	+7.0
7.5	7.92	1.1	+5.6

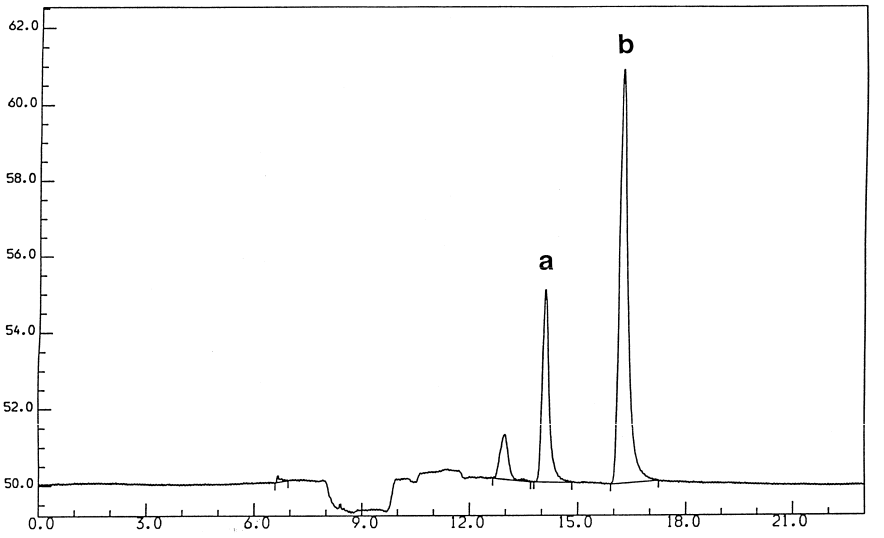

Fig. 7. Extracted sample from a dosed volunteer. (a) EFZ, (b) I.S.

4. Conclusions

An automated SPE, heart-cut column-switching HPLC was developed and validated to quantify EFZ in human plasma and urine. This method was applied to the analysis of samples from clinical studies to provide data on bioavailability and pharmacokinetics of EFZ (Fig. 7). This method allows one hundred plasma samples to be processed every 2.75 h on a 10-module RapidTrace® workstation with minimal analyst attention. Urine samples required no extraction at all. The method was shown to be selective and rugged throughout the method validation and application during four pharmacokinetic studies including 3800 plasma and 1200 urine samples.

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