ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
26 (2001) 665–674

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Gas chromatographic assay of diethylcarbamazine in human plasma for application to clinical pharmacokinetic studies

Joseph R. Miller Jr., Lawrence Fleckenstein *

College of Pharmacy, The University of Iowa, S-427 Pharmacy, Iowa City, IA 52242, USA

Received 27 October 2000; received in revised form 12 March 2001; accepted 15 March 2001

Abstract

A sensitive and selective gas chromatography method using flame ionization detection was developed for the determination of diethylcarbamazine (DEC) in human plasma. DEC and the internal standard, 1-diethylcarbamyl-4-ethyl piperazine HCl (E-DEC), were extracted from human plasma after loading onto a conditioned C_{18} solid phase extraction cartridge, rinsed with water and eluted with methanol. After evaporation under a stream of nitrogen and reconstitution in methanol, 3 μl were injected onto the GC system. Separation was achieved on a A Heliflex® AT-35 capillary column (length 30 m, internal diameter 0.32 mm). Gas flow rates were: hydrogen, 35 ml/min; carrier gas (helium), 1.5 ml/min, make-up gas (helium), 25 ml/min; and air 420 ml/min. The retention times of DEC and internal standard were approximately 5.5 and 7.28 min, respectively. The GC run time was 22 min. The assay was linear in concentration range 100–2000 ng/ml for DEC in human plasma. The analysis of quality control samples for DEC (120, 1000, 2000 ng/ml) demonstrated excellent precision with coefficients of variation of 4.5, 1.3, and 1.6%, respectively ($n = 6$). The method was accurate with all intra-day ($n = 6$) and inter-day ($n = 12$) mean concentrations within 4.3% from nominal at all quality control sample concentrations. DEC was found to be stable after 3 freeze-thaw cycles, and with storage at −20°C for 12 weeks. The method is currently being used for pharmacokinetic studies of DEC in healthy volunteers.

Keywords: Diethylcarbamazine; Antifilarial; Plasma; Gas chromatography

1. Introduction

Single doses of DEC in combination with albendazole has been found safe and efficacious for the treatment of *Brugia malayi* infection [1], and single dose treatment of 6 mg/kg of DEC with ivermectin is effective against adult *Wuchereria bancrofti* [2]. These and other studies support mass treatment campaigns for the elimination of lymphatic filariasis. Despite numerous DEC efficacy trials for the treatment of lymphatic filariasis, the pharmacokinetics of DEC in various body fluids has not been well characterized. Spectroscopic methods of analysis that utilize chromophores, like HPLC with UV detection, are not suitable for DEC because it is a compound that lacks a chromophore. A major barrier to the investigation of DEC pharmacokinetics is the lack

* Corresponding author. Tel.: +1-319-3358804; fax: +1-319-3535646.

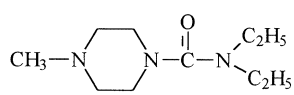
E-mail address: l-fleckenstein@uiowa.edu (L. Fleckenstein).

0731-7085/01/$ - see front matter
PII: S0731-7085(01)00435-6

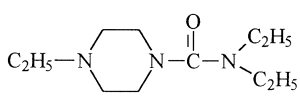
of a specific and sensitive analytical method for biological fluids.

In order to achieve the sensitivity and specificity for the determination of DEC in plasma, a number of gas chromatographic methods have been developed [3–7]. We previously reported a sensitive and specific gas chromatography method that used solid phase extraction and nitrogen–phosphorus detection [7]. Regular maintenance that was required for the nitrogen–phosphorus detector made it difficult to apply this method to clinical pharmacokinetic studies with high sample throughput requirements.

In this study, we report a sensitive, selective, accurate and reproducible gas chromatographic assay using flame ionization detection for the determination of DEC in human plasma. With the use of an autoinjector, this method is suitable for processing a large number of DEC plasma samples withdrawn during clinical pharmacokinetic studies.



Diethylcarbamazine



1-Diethylcarbamyl-4-ethylpiperazine

Fig. 1. Structures of diethylcarbamazine and the internal standard, 1-diethylcarbamyl-4-ethyl piperazine.

Table 1
Calibration curve parameters and statistics for DEC in human plasma[a]

Curve	Slope	*y*-intercept	Correlation coefficient
1	1.019E−03	1.123E−02	0.9976
2	1.012E−03	9.915E−03	0.9982
3	1.028E−03	1.099E−02	0.9996
4	1.008E−03	2.249E−02	0.9989
5	9.894E−04	2.997E−02	0.9986
6	9.725E−04	1.092E−02	0.9983
Mean ($n=6$)	1.005E−03	1.592E−02	0.9985
S.D.	2.041E−05		
CV(%)	2.03		

[a] S.D., standard deviation; CV, coefficient of variation.

2. Experimental

2.1. Solvents and chemicals

All solvents and chemicals were HPLC grade. Organic solvents, sodium carbonate (anhydrous), and sodium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Carbonate buffer, pH 10.0, was prepared using the sodium carbonate (anhydrous), and sodium bicarbonate. A solution of 0.1% triethylamine in methanol was made for the elution solvent. Diethylcarbamazine citrate was obtained from Sigma, and used to make stock solutions (St. Louis, MO, USA). The internal standard, 1-diethylcarbamyl-4-ethylpiperazine (E-DEC) was synthesized by the Division of Medicinal and Natural Products Chemistry at the University of Iowa, College of Pharmacy. Ultrapure analytical grade Type I water was produced by a Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA).

2.2. Standard stock solutions

Diethylcarbamazine citrate was used to make stock solutions. Diethylcarbamazine citrate, and E-DEC were weighed on a Mettler-Toledo AG104 analytical balance (Mettler-Toledo, Inc., Hightstown, NJ, USA). The weight of DEC citrate was corrected for the salt to measure the concentration of DEC. The appropriate amounts of drug were dissolved using methanol in volumetric flasks to make a 1 mg/ml stock solution of each. A working standard for each concentration point on the standard curve was prepared, as well as a 21 μg/ml working solution for the internal standard. For all quantitated samples, 25 μl of working internal standard solution was added. All standard solutions were stored in a −20°C

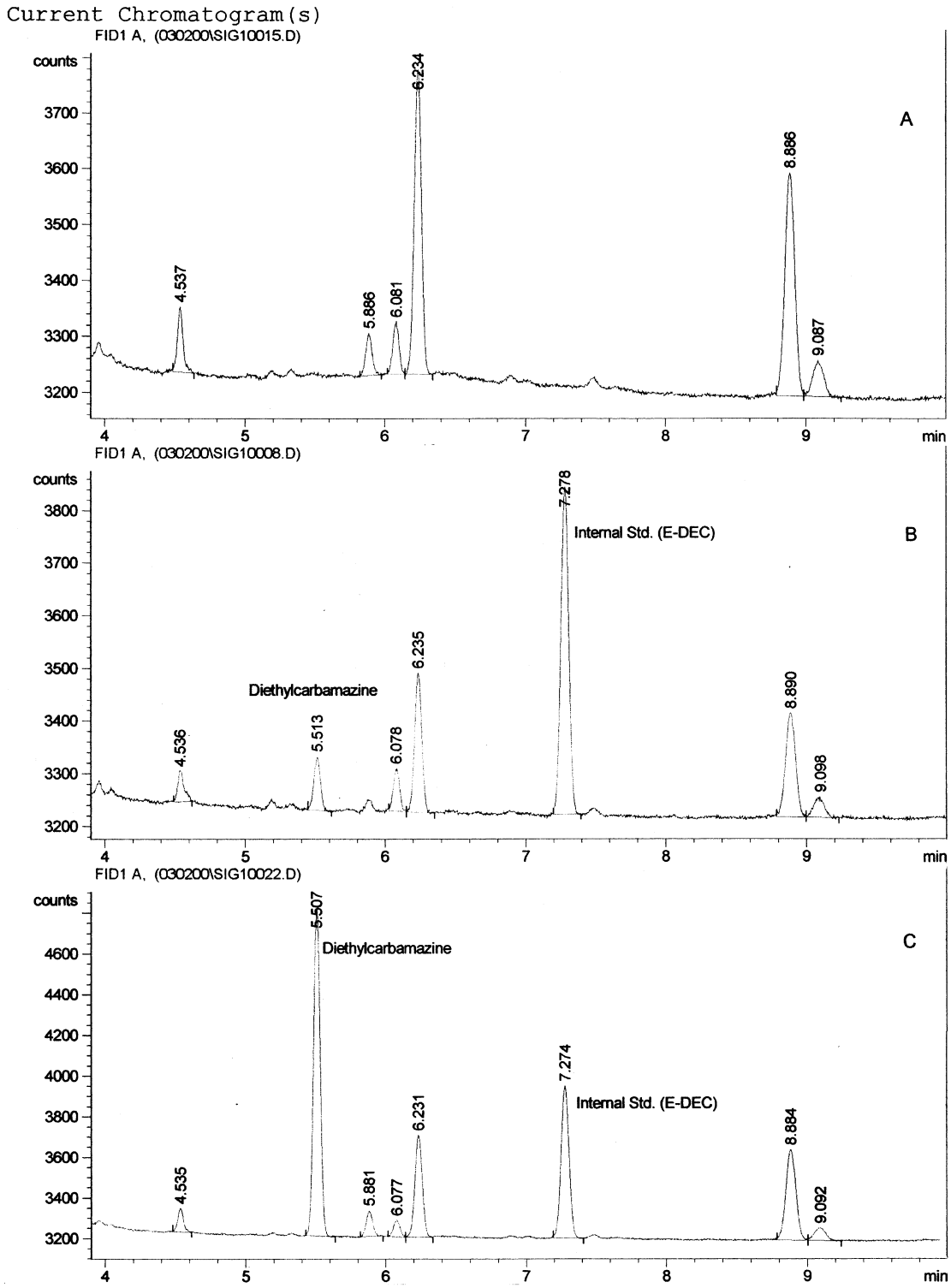


Fig. 2. Chromatograms of (A) blank (analyte-free) human plasma; (B) calibration standard containing 120 ng/ml DEC in human plasma; and (C) calibration standard containing 1700 ng/ml DEC in human plasma.

Table 2
Evaluation of the lower limit of quantitation (70 ng/ml) for diethylcarbamazine in human plasma[a]

Sample number	Calculated concentration (ng/ml)	DEV (%)
1	67.3	−3.86
2	63.8	−8.86
3	67.3	−3.86
4	70.0	0.00
5	62.9	−10.14
6	62.9	−10.14
Within day mean ($n = 6$)	65.7	
S.D.	2.93	
CV (%)	4.46	
DMT (%)	−6.14	

[a] S.D., standard deviation; CV, coefficient of variation; DMT, deviation of mean value from nominal; DEV, deviation of single value from nominal.

freezer, and fresh stock solutions were made monthly, or as needed.

2.3. Instrumentation

Chromatographic separations were performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph equipped with a flame ionization detector (FID) and a HP 7673 automatic sampler (Hewlett-Packard, Palo Alto, CA, USA). Integration results from this automated system were processed by using HP ChemStation Rev. A.06.03 and a Hewlett-Packard DeskJet 812C printer. The GC was operated at the following temperatures: injector, 180°C; oven, 160°C; and the detector at 240°C. Gas flow rates were: hydrogen, 35 ml/min; carrier gas (helium), 1.5 ml/min, make-up gas (helium), 25 ml/min; and air 420 ml/min. The carrier gas was passed through an Alltech gas purifier and Oxy-Trap® before entering the GC. The column head pressure was 11 p.s.i.. A Heliflex® AT-35 capillary column, 30 m long with an internal diameter of 0.32 mm and a film thickness of 0.25 μm was used (Alltech, Deerfield, IL, USA). Compressed air, helium, and hydrogen were obtained from the University of Iowa general stores (Iowa City, IA, USA).

2.4. Extraction procedure

This procedure was validated using 0.5 ml of spiked human plasma. Outdated human plasma was obtained from the Blood Bank at the University of Iowa Hospitals and Clinics and stored frozen in aliquots at −20°C. Extraction of the DEC and internal standard was carried out by solid phase extraction (SPE). Alltech Extract — Clean® C_{18} cartridges, 500 mg with a 2.8 ml reservoir, and a SPE vacuum manifold (Alltech, Deerfield, IL, USA) were used for the procedure. Pipetman® precision microliter pipettes (Woburn, MA, USA) were used throughout the assay.

Table 3
Intra-day and inter-day precision and accuracy for DEC in human plasma[a]

	Theoretical concentration (ng/ml)		
	120	1000	2000
Intra-day run			
Overall mean ($n = 6$)	116	1021	2028
S.D.	5.2	13.3	32
CV (%)	4.5	1.3	1.6
DMT (%)	−4.3	2	1.4
Inter-day run			
Overall mean ($n = 12$)	118	1026	2016
S.D.	8.2	14.2	33.2
CV (%)	6.9	1.4	1.6
DMT (%)	−1.7	2.6	0.8

[a] S.D., standard deviation; CV, coefficient of variation; DMT, deviation of mean value from nominal.

Table 4
Recovery of DEC and E-DEC (internal standard from human plasma)

Concentration (ng/ml)	DEC			E-DEC (internal standard)		
	Mean extract area ($n = 6$)	Mean pure area ($n = 6$)	Mean % recovery ($n = 6$)	Mean extract area ($n = 6$)	Mean pure area ($n = 6$)	Mean % recovery ($n = 6$)
120	338	372	90.9	2685	2961	90.7
CV (%) ($n = 6$)	6.8	5.7		5.3	7.8	
1000	3312	4035	82.1	3206	3893	82.4
CV (%) ($n = 6$)	12.9	5.7		12.0	4.8	
2000	8275	10 183	81.3	4059	4866	83.4
CV (%) ($n = 6$)	11.9	16.9		12.1	17.6	
Overall recovery ($n = 18$)			84.8			85.5

Table 5
Autosampler stability of a DEC (600 ng/ml) extracted human plasma sample[a]

Time (h)	Calculated concentration (ng/ml)
0	631
1.63	632
2.87	636
4.10	632
5.33	634
6.57	626
7.80	632
9.45	641
13.35	637
Mean	633
S.D.	4.15
CV%	0.7

[a] S.D., standard deviation; CV, coefficient of variation.

Table 6
Freeze and thaw stability of DEC in plasma specimens during 3 freeze-thaw cycles[a]

Time period	Theoretical concentration (ng/ml)	
	120	2000
Time = 0	$n = 5$	$n = 5$
Mean	137	2056
S.D.	8.4	41.6
CV (%)	6.1	2.0
Cycle 2	$n = 5$	$n = 5$
Mean	152	1851
S.D.	9.9	17.3
CV (%)	6.5	0.9
Cycle 3	$n = 5$	$n = 4$
Mean	122	2114
S.D.	8.9	351
CV (%)	7.3	16.6

[a] S.D., standard deviation; CV, coefficient of variation.

An eight point calibration curve was prepared daily with each set of samples by adding 25 μl of the appropriate DEC working solution, and 25 μl of internal standard working solution to 0.5 ml of human plasma. Prior to extraction 1.0 ml of carbonate buffer pH 10.0 was added to all samples, and vortex mixed for 30 s. For each sample a SPE cartridges was activated by aspirating 1 cartridge volume of methanol, followed by 1 cartridge volume of HPLC grade water. The samples were then transferred to the appropriate activated cartridge, and aspirated at a reduced vacuum (5–7 in Hg.) After aspiration, each cartridge was washed with 2.5 ml of HPLC grade water, and allowed to dry for 10 min under vacuum. For sample elution, 2 ml of 0.1% triethylamine in methanol was added to each cartridge, and allowed to pass through the cartridge into properly labeled 5 ml disposable centrifuge tubes under a low vacuum. After elution, the eluant was evaporated in a 40°C N-EVAP® (Berlin, MA, USA), under a gentle stream of nitrogen. When evaporation was complete samples were reconstituted with 50 μl of methanol, and vortex mix for 60 s. The reconstituted samples were transferred to vials containing limited volume conical inserts and placed on the autosampler where 3 μl was injected into the GC.

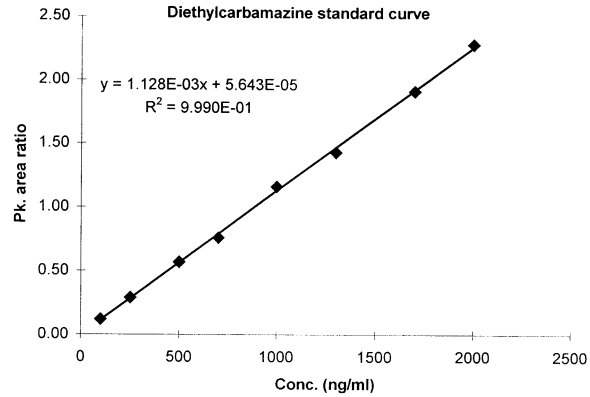


Fig. 3. Plot of typical DEC calibration curve.

2.5. Data regression

Chromatographic data were collected and integrated by the ChemStation chromatography system. Peak area ratios of DEC/E-DEC were calculated. The calibration curves were obtained by unweighted least-squares linear regression analysis. The equations of the calibration curves were then used to calculate the concentrations of DEC in the samples and QC samples by their peak area ratios.

2.6. Precision, accuracy, and recovery

Precision, accuracy, and recovery were evaluated by conducting repeated analysis ($n = 6$) of spiked plasma samples at three different concentration levels: 120, 1000, and 2000 ng/ml. The coefficient of variation of each concentration was calculated to determine the precision of the method. Comparing the measured concentrations of extracted plasma samples, and the true concentration in the spiked samples determined the accuracy of the method. Recovery was assessed by comparing the detector response from an amount of DEC added to and extracted from drug free human plasma, compared to the detector response obtained for unextracted standard mixtures representing 100% recovery.

2.7. Stability

Freeze and thaw stability, long-term stability, and autosampler stability experiments were con-

Table 7
Storage stability of 120 and 2000 ng/ml DEC in human plasma at −20°C[a]

Time (weeks)	0	2	4	9	12
120 ng/ml					
Mean ($n = 6$)	100	136	108	86	99
S.D.	13.0	18.7	22.8	8.4	3.7
CV (%)	13.0	13.8	21.1	9.7	3.7
2000 ng/ml					
Mean ($n = 6$)	1996	1970	1980	1930	2070
S.D.	16.7	96.6	37.8	27.0	195
CV (%)	0.8	4.9	1.9	1.4	9.4

[a] S.D., standard deviation; CV, coefficient of variation.

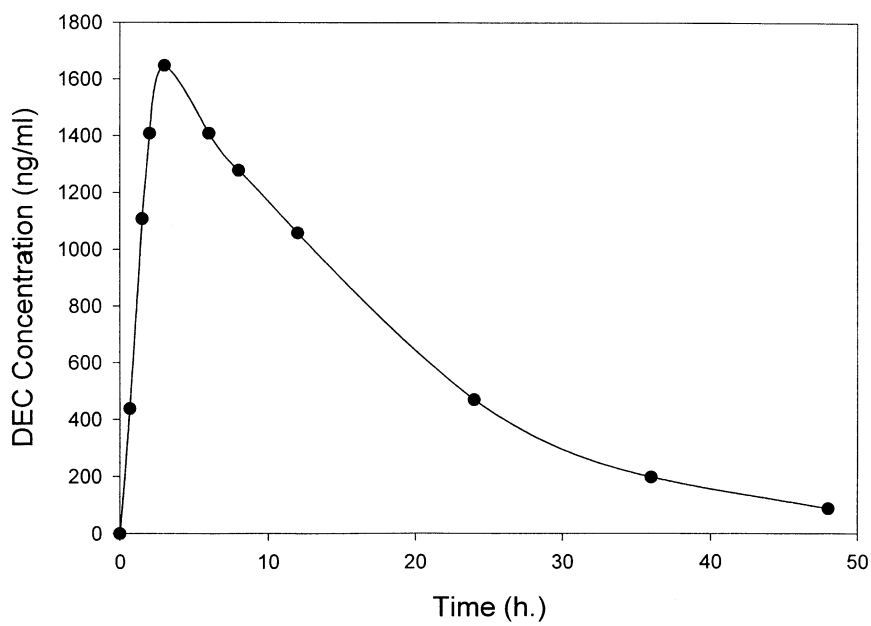


Fig. 4. Plasma concentration-time profile for DEC in a healthy subject after taking 300 mg DEC citrate orally.

ducted. The long-term stability experiment of DEC in human plasma was assessed over a period of 12 weeks. Both the freeze/thaw and long-term stability studies were carried out at two concentration levels by spiking drug-free plasma to 120 and 2000 ng/ml with storage in a −20°C freezer (Model ULT 2540-7-A12 freezer, Revco Scientific Inc., Asheville, NC, USA). The freeze/thaw experiment was carried out over 3 freeze and thaw cycles. Autosampler stability was conducted under the usual conditions by repeated injection of an extracted spiked 600 ng/ml plasma sample.

3. Results and discussion

3.1. Separation

The molecular structures of DEC and E-DEC (internal standard) are shown in Fig. 1. Sample chromatograms are shown in Fig. 2. Blank plasma from six separate lots of human plasma was tested for endogenous interference. Of the lots tested, all were clear of interference in the DEC and internal standard regions. A representative chromatogram of the plasma blank selected for use in the validation procedure is shown in Fig. 2A. Fig. 2B and C show chromatograms of calibration standards containing 120–1700 ng/ml of DEC in human plasma, respectively. The retention time for DEC is 5.5 min and for the internal standard (E-DEC) it is 7.28 min. The HP ChemStation and autosampler allow a unattended injection of multiple samples, with a reasonable run time of approximately 22 min per sample.

3.2. Linearity

Calibration curve parameters for DEC are shown in Table 1. A plot of a typical standard curve is shown in Fig. 3. Results were calculated using peak area ratios. Calibration curves for DEC in human plasma were linear using unweighted linear regression in the concentration range of 100–2000 ng/ml, with correlation coefficients greater than or equal to 0.9934 for all curves. The calibration curve covers a range up to 150% of the expected concentrations for analyzing the body fluids of subjects given a 6 mg/kg oral dose of DEC citrate.

The limit of quantification (LOQ) in human plasma was accepted as 70 ng/ml. Plasma samples were spiked to a nominal concentration of 70 ng/ml with DEC working solution and internal standard and carried through the extraction procedure. At the LOQ, the C.V. ($n = 6$) of the measured concentration was 4.5%, and the deviation of the mean of the measured concentrations from the nominal value was −6.1% (Table 2). The limit of detection, defined as a signal to noise ratio of 5:1 is 60 ng/ml. Increased sensitivity can be achieved, if needed, by using 1 ml of plasma.

3.3. Precision, and accuracy

Data on precision and accuracy are shown in Table 3. For an intra-day run ($n = 6$), the coefficient of variation of DEC at 120, 1000, and 2000 ng/ml has been shown to be 4.5, 1.3, and 1.6%, respectively. The deviation of mean values from nominal ($n = 6$) are −4.3, 2 and 1.4% for DEC concentrations 120, 1000, and 2000 ng/ml, respectively. The inter-day precision is also shown in Table 3. The CV results for inter-day precision at the same concentrations are all less than 7% ($n = 12$). The deviation of mean values from nominal ($n = 12$) are −1.7, 2.6 and 0.8% for DEC concentrations 120, 1000, and 2000 ng/ml, respectively. These results indicate there is good reproducibility and accuracy for the determination of DEC for samples determined on the same or different days.

3.4. Absolute recoveries

Recovery was tested at low, medium and high concentrations of DEC and internal standard. Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for DEC and the internal standard were 84.8 and 85.5%, respectively (Table 4).

3.5. Stability

Autoinjector stability was carried out for over 13 h by repeated injection of the same extracted plasma sample at room temperature (nominally 25°C). The results presented in Table 5 show the extracted specimens remained stable over the course of the study.

QC samples containing 120 and 2000 ng/ml DEC in plasma were subjected to 3 freeze/thaw cycles. Samples were frozen at −20°C for 24 h and thawed unassisted at room temperature. When completely thawed the samples were transferred back to the original freezer and kept refrozen at least 24 h. Freezing and thawing of the QC samples appeared to have no effect on quantitation of the analyte (Table 6).

QC samples containing 120 and 2000 ng/ml DEC in plasma were subjected to storage at −20°C for 12 weeks. Plasma samples ($n = 6$) were taken for DEC analysis at 0, 2, 4, 9 and 12 weeks (Table 7). The QC samples stored in a freezer set to maintain −20°C remained stable for the duration of the study period.

3.6. Application of assay

Fig. 4 shows the concentration time profile of DEC in plasma for a human subject. The concentrations were measured using the described assay using samples obtained from a healthy subject after oral administration of 300 mg of DEC citrate. Control samples with nominal concentration of 200, 1000 and 1660 ng/ml were analyzed at the beginning and the end of the analytical run.

3.7. Interference by other drugs

We have tested the assay for potential interference by other drugs and metabolites that may interfere with the assay. We have tested EDTA (used as an anticoagulant), albendazole and its metabolite, albendazole sulfoxide, spiked into plasma samples carried through the assay procedure and found no interference. We also tested for potential interference by the DEC metabolite, DEC-N-oxide [8–10]. DEC-N-oxide is a potential concern, because the N-oxide is thermally labile and can be converted back to DEC under the elevated temperature conditions of the gas chromatograph [7]. However, DEC-N-oxide does not interfere with the assay because it is removed by the solid phase extraction procedure.

4. Conclusions

We describe a gas chromatographic assay procedure using solid phase extraction for the specific and quantitative analysis of DEC in human plasma samples. The assay uses 1-diethylcarbamyl-4-ethylpiperazine as an internal standard and has a run time of approximately 22 min. The assay has been validated and the results of validation demonstrate that the standard curve is linear over the concentration range of 100–2000 ng/ml. The assay is reproducible and accurate, with recovery of the analyte and internal standard in the range of 80–90%. The analysis requires 0.5 ml of plasma and has a detection limit of 60 ng/ml. The stability of plasma samples stored at −20°C has been demonstrated for up to 12 weeks. Autoinjector stability has been demonstrated for over 13 h and freeze-thaw stability has been demonstrated for 3 freeze-thaw cycles. The procedure has a sample throughput of at least 30 specimens per day. The assay meets the guidelines for bioanalytical methods validation for human studies [11].

Acknowledgements

This investigation was supported by the Onchocerciasis Control Program in West Africa-UNDP/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases Macrofil Chemotherapy Project.

References

[1] R.K. Shenoy, S. Dalia, A. John, T.K. Suma, V. Kumaraswami, Ann. Trop. Med. Parasit. 93 (1999) 643–651.
[2] G. Dreyer, D. Addiss, A. Santos, J. Figueredo-Silva, J. Noroes, Trans. Royal Soc. Trop. Med. Hyg. 92 (1998) 219–222.

[3] J.A. Bogan, Analyst 102 (1977) 56–61.
[4] G.D. Allen, T.M. Goodchild, B.C. Weatherley, J. Chromatogr. 164 (1979) 521–526.
[5] S. Nene, B. Anjaneyulu, T.G. Rajagopalan, J. Chromatogr. 308 (1984) 334–340.
[6] G. Edwards, K. Awadzi, A. Breckenridge, H.M. Gilles, M.L. Orme, S.A. Ward, Clin. Pharmacol. Ther. 30 (1981) 551–557.
[7] S. Lee, D.A. Casteel, L. Fleckenstein, J. Chromatogr. B 704 (1997) 181–185.
[8] D.R. Bangharn, Br. J. Clin. Pharmacol. 10 (1955) 397–405.
[9] B. Chandrasekaran, B.C. Harinath, Indian J. Exp. Biol. 18 (1980) 722–724.
[10] R.K. Chatterjee, N. Fatma, V.K. Agarwal, S. Sharma, N. Anand, Trop. Med. Parasitol. 40 (1989) 474–475.
[11] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. Mcdowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249–255.