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Analysis of Diethylcarbamazine and Diethylcarbamazine-*N*-oxide by Gas Chromatography

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Diethylcarbamazine (DEC, 1-diethylcarbamyl-4-methylpiperazine) is an antiparasitic piperazine derivative used in the treatment of lymphatic filariasis caused by *Wuchereria bancrofti, Brugia malayi* or *Brugia timori.* DEC-*N*-oxide is a major metabolite in humans and has antifilarial activity. In carrying out pharmacokinetic studies, gas chromatographic analysis of DEC in plasma can be complicated by the presence of the metabolite, since the thermally unstable DEC-*N*-oxide is converted back to a material which coelutes with DEC under the conditions of the analysis. We now report a method to separate DEC-*N*-oxide from DEC in plasma using solid phase extraction with subsequent gas chromatographic analysis using a nitrogen specific detector. One-diethylcarbamyl-4-ethylpiperazine (E-DEC) was the internal standard. The standard curve of DEC was linear in the range of 10 to 200 ng/ml as described by $Y=0.0350+0.0128X$, $R^2=0.999$. The limit of quantitation was 4 ng/mL. Reproducibility at 10, 100 and 200 ng/mL concentration points of the standard curve gave coefficient variations of 6.1%, 7.8% and 1.6%, respectively. The recovery following solid phase extraction was 99.3% for DEC and 94.8% for the internal standard. This sensitive and specific analytical method is suitable for pharmacokinetic studies of DEC.

Key words : Diethylcarbamazine, Diethylcarbamazine-*N*-oxide, Antifilarial activity, Filariasis-lymph, Gas chromatographic analysis

INTRODUCTION

Diethylcarbamazine, 1-diethylcarbamyl-4-methylpiperazine (DEC), has been widely used in the treatment and control of human filariasis (Hawking, 1976, Kimura *et al.*, 1992). Its *N*-oxide metabolite has been reported to be a major and active metabolite in mammals including humans (Bangham, 1955, Chandrasekaran *et al.*, 1980, Chatterjee *et al.*, 1989). Gas chromatography (GC) has been used for analysis of diethylcarbamazine (Bogan, 1977, Allen *et al.*, 1979, Nene *et al.*, 1984).

Several pharmacokinetic studies have been done with analysis of diethylcarbamazine (DEC) in palsma and diethylcarbamazine-*N*-oxide (DEC-*N*-oxide) in urine using gas chromatography (Edwards *et al.*, 1981a,b). The DEC-*N*-oxide has been reported to be a major and active metabolite in mammals, including humans (Chatterjee *et al.*, 1989). One of pharmacokinetic studies showed plasma concentration-time curves with a secondary rise of DEC concentration at 8 to 10 hours later after administration of DEC citrate (Edwards *et al.*, 1981a). There was no clear explanation for this pharmacokinetic observation although the possibility of enterohepatic recirculation was mentioned. The specific measurement of DEC by separation of DEC-*N*-oxide from DEC in plasma can be in error if the separation is not complete. It is concerned that DEC-*N*-oxide may be thermally unstable during GC analysis, and in the presence of the metabolite in plasma, conversion to DEC may result in falsely elevated DEC concentrations (Edwards *et al.*, 1981a , Faulkner *et al.*, 1972). Therefore, the specific measurement of DEC with separation of DEC-*N*-oxide is required for pharmacokinetic studies of DEC.

We now report a method of separating DEC-*N*-oxide from DEC in plasma using solid phase extraction with subsequent specific DEC assay by GC analysis using a nitrogen specific detector. Physicochemical properties of the compounds of interest were characterized by differential scanning thermal analysis and pK_a determinations.

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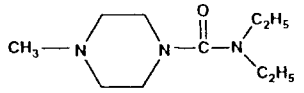
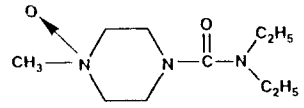
MATERIALS AND METHODS

Materials

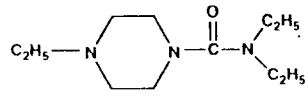
Diethylcarbamazine citrate were obtained from Sigma Chemical Company (St. Louis, MO, USA). Diethyl-



Diethylcarbamazine



Diethylcarbamazine-*N*-oxide



1-Diethylcarbamyl-4-ethylpiperazine

Fig. 1. Chemical structures of the compounds

carbamazine-*N*-oxide fumarate and 1-diethylcarbamyl-4-ethylpiperazine HCl (E-DEC, internal standard) were synthesized and the structures were confirmed on the basis of IR and NMR spectra (Kushner *et al.*, 1948, Morren, 1955). Fig. 1 shows the chemical structures of the compounds. KOH pellets, methylene chloride, anhydrous sodium sulfate, benzene, potassium carbonate, acetone, benzene, chloroform, ethylpiperazine and diethylcarbamylchloride were purchased Sigma Chemical Company or Aldrich Chemical Company (Milwaukee, WI, USA). All chemicals and solvents used were reagent grade or chromatography quality. Solid phase extraction cartridges were C_{18}, 500 mg with 2.8 mL reservoir volume (Baxter Healthcare Corporation, Burdick and Jackson Division, Muskegon, MI, USA). Double-distilled water was made by Mega-Pure System MP-1 (Corning, NY, USA).

pK_a assessment

The pK_a titration was performed for 1 mM solutions of diethylcarbamazine free base, and diethylcarbamazine-*N*-oxide free base with 0.1 N NaOH at the controlled temperature of 37°C. A Metrohm 713 pH meter, a Metrohm Multi-Dosimet-Model 665, and Brinkman combination electrode were used.

Thermal analysis

A Perkin-Elmer Differential Scanning Calorimeter (DSC-2) and Thermal Analysis Data System (TADS) were used for determining thermal properties of the solid compounds. One to 3 mg of DEC citrate, DEC-*N*-oxide fumarate and E-DEC HCL were weighed in Perkin-Elmer aluminum sample pan with a Cahn C-31 microbalance. The sample and the reference pan were simultaneously heated at 10°C/min from 30°C to 300°C. Thermograms of differential heat input versus temperature with amplified energy transition area were used to determine the thermal stability and melting points of each compound (Willard *et al.*, 1988).

Gas chromatography

A Hewlett Packard Gas Chromatograph 5890A equipped with nitrogen and phosphorous detector (NPD) was used for the analysis. It was operated at the following temperatures: column, 160°C; injector, 180°C and detector, 240°C. The maximum oven temperature was 225°C. Gas flow rates were: hydrogen, 3 ml/min; helium, 25 ml/min and air, 125 ml/min. The column head pressure was 35 psig. The glass packed column was 6 feet long and the internal diameter was 2 mm (Alltech 9855A, configuration A). The packing material was 2% Carbowax 20 M and 5% KOH on Chromosorb GAW/DMCS (mesh 100/120) support. The parameters for the detector were; range 1, attenuation 0 and zero 10. The standing current was adjusted to the range of 15-20 pA with an NPD bead power of 450. A Hewlett Packard 3390A integrator was set to 1 mV full scale. The integration parameters were: attenuation, 5 (2^5); chart speed, 0.5 cm/min; peak width, 0.16 minutes; threshold, 5; area rejection, 400; run time, 20 minutes.

Solid phase extraction

The required number of extraction cartridges were placed on the vacuum column processor. The following procedures were applied to clean up the plasma sample and separate DEC-*N*-oxide from DEC in plasma with solid phase extraction: 1) prepare DEC citrate, DEC-*N*-oxide fumarate and E-DEC HCL 20 mM solutions; 2) 100 µl of each solution was mixed with 900 µl of pH 10 carbonate buffer and 1000 µl of plasma for final concentration of 400 µg/ml; 3) condition the cartridge with 3 ml methanol and 3 ml water, eluting the liquid each time with suction using a vacuum of -20 kPa without letting it dry until next step; 4) load 1 ml of the sample on the cartridges; 5) mild suction was applied so that the sample solution passed through the cartridges at a flow rate of approximately 0.5 ml/min; 6) wash the cartridge by eluting with 3 ml water and dried at vacuum of -20 kPa for 10 min; 7) elute twice with 1 ml methanol containing 0.1% triethylamine; 8) evaporate the eluant to dryness under N_2 gas; 9) reconstitute the dried residue with 100 µl methanol; and 10) inject 1 µl aliquot into GC-NPD system.

Recovery was assessed by comparison of pre-extraction samples with post-extraction samples. DEC citrate and E-DEC HCl in methanol were injected onto GC/NPD for determination of pre-extraction DEC

area. Samples of the same concentration were injected after solid phase extraction and recovery was calculated.

DEC assay

Samples were prepared in the range of 10 ng/ml to 200 ng/ml of DEC in plasma using the same procedure as for solid phase extraction. Peak area ratios of DEC to E-DEC, the internal standard, were used to construct the standard curve using linear regression. Six repeated DEC samples were prepared at three concentration points that were 10 ng/ml, 100 ng/ml and 200 ng/ml. The rest of the procedure was the same as that used for the standard curve. The peak area ratios of each concentration were analyzed statistically for reproducibility. The measured concentrations were analyzed statistically for accuracy with the same three concentrations as that for reproducibility.

RESULTS AND DISCUSSION

pK_a assessment

The free bases of DEC and DEC-*N*-oxide were titrated instead of DEC citrate and DEC-*N*-oxide fumarate because the pK_a's of citric or fumaric acids might overlap with those of DEC and DEC-*N*-oxide. The pH of sample solutions increases as the titrant, 0. 1 N NaOH, is added in small amounts (Fig. 2). A plot of pH vs. G has the same tendency. The function of G is defined as the following equation,

$$G=V_t+[(V_o+V_t)(\{H^+\}-\{OH^-\})]/N$$

where V_t is the volume of the titrant added, V_o is the initial volume of DEC and DEC-*N*-oxide solution to be titrated and N is the normality of the titrant, 0.1 N NaOH at 37°C. The determined pK_a's were 7.37 for DEC and 7.00 for DEC-*N*-oxide. Since the pK_a's were so close, these results discouraged us from exploring differential extraction to separate DEC-*N*-oxide from DEC.

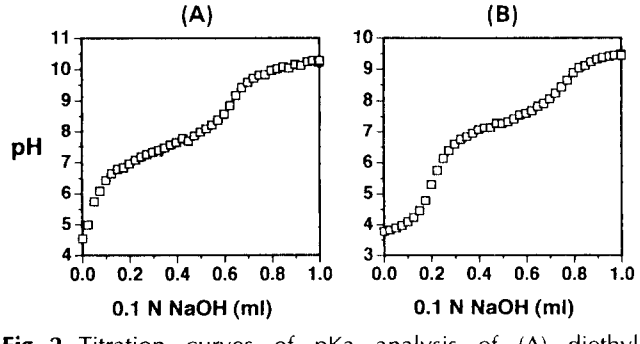


Fig. 2. Titration curves of pKa analysis of (A) diethylcarbamazine, (B) diethylcarbamazine-*N*-oxide

Thermal analysis

Thermal analysis defined the thermal stability of DEC citrate, DEC-*N*-oxide fumarate and 1-diethylcarbamyl-4-ethylpiperazine (E-DEC) HCl for the high temperature conditions of GC analysis. Fig. 3 shows the differential scanning calorimetry thermograms for DEC citrate, E-DEC HCl and DEC-*N*-oxide fumarate. The onset of melting for DEC citrate was found to be 136.4°C and 188.6°C for E-DEC HCl: both were thermally stable as the solids and the melted. On the other hands, the differential scanning calorimetry thermogram for DEC-*N*-oxide fumarate showed melting from 127.9°C after which it underwent chemical decomposition. The thermal analysis data indicated that DEC-*N*-oxide was thermally unstable and might decompose under the conditions of the GC analysis.

Gas chromatography

The retention time were 5.7 min for DEC and 7.5 min for E-DEC (Fig. 4). The relative retention time between DEC and E-DEC was 1.33. Blank plasma samples after solid phase extraction did not have any interfering peaks around the retention times of DEC or E-DEC, the internal standard. DEC had a single peak, but DEC-*N*-oxide had a peak at the retention time of DEC and a second peak later, which appeared at irregular time . These data indicated that a part of DEC-*N*-oxide was converted back to DEC or a material which coeluted with DEC. There was an additional large peak at 10.9 min that did not interfere with the peaks of DEC or E-DEC. This peak was an unidentified material which coeluted from the solid phase extraction cartridge. Since the extra peak only caused an extension of the analysis time without any interference with the peaks of DEC and E-DEC, further attempts to remove it were not pursued.

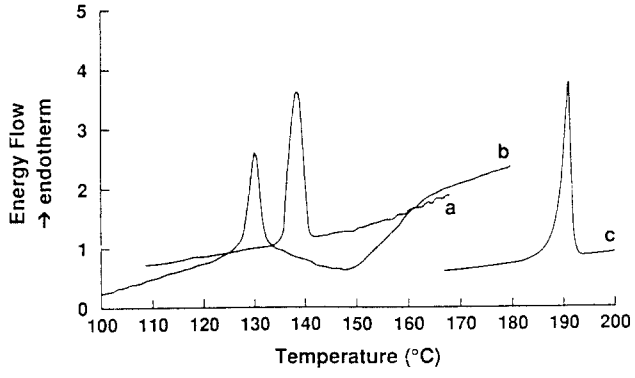


Fig. 3. Thermal analysis of a) diethylcarbamazine citrate, mp 136.4°C; b) diethylcarbamazine-*N*-oxide fumarate decomposed after melting, mp 127.9°C; c) 1-diethylcarbamyl-4-ethylpiperazine hydrochloride, mp 188.6°C

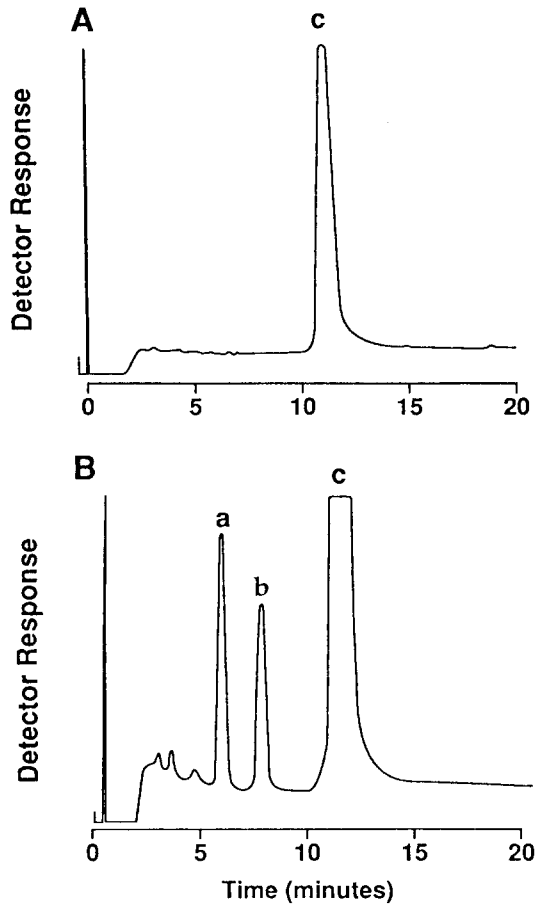


Fig. 4. Chromatograms after solid phase extraction and GC analysis of (A) plasma blank, (B) samples in plasma (80 ng/ml): a) diethylcarbamazine, 5.7 min, b) 1-diethylcarbamyl-4-ethylpiperazine, 7.5 min, c) a unidentified coeluant, 10.9 min

Solid phase extraction

Reverse phase extraction cartridges were effective in cleaning up the plasma samples and separation of DEC-*N*-oxide from DEC and E-DEC in plasma. E-DEC had similar extraction properties as DEC which is an ideal characteristic for an internal standard. The capacity of the sorbent (500 mg) was large enough to handle a plasma sample which has other materials than the analytes. Reservoir size (2.8 ml) was large enough to condition and rinse the cartridge appropriately. DEC-*N*-oxide did not elute at any stage which implies that it is retained in the cartridge and separated from DEC by reverse phase system. The mechanism of separation using solid phase extraction could be explained by pKa difference and chromatographic properties. Recovery was 99.3% for DEC and 94.8% for E-DEC, which was considered satisfactory.

DEC assay

A standard curve of peak area ratio vs. DEC concentration was linear in the range of 10 ng/ml to 200 ng/ml when we used solid phase extraction and gas chromatography with a nitrogen specific detector. The range of concentration was corresponding to the serum concentration level for 48 hours after 0.5 mg/kg of DEC citrate was taken by patients. The lowest concentration measured was 4 ng/ml, which was a higher concentration than the detection limit and had a signal to noise ratio greater than 5. There was also the possibility of increasing the assay sensitivity by using a larger plasma sample, reconstituting in a smaller volume and/or injecting more sample. The standard curve was described as $Y=0.035+0.0128X$, $R^2=0.999$, where Y was the peak area ratio and X was DEC concentration (ng/ml). The coefficient variations for the reproducibility were 6.1% for 10 ng/ml, 7.8% for 100 ng/ml and 1.6% for 200 ng/ml. Acceptable ranges were defined according to a summary report of the conference on "Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies" (Shah *et al.*, 1992). All data were included in the acceptable range as mean$\pm$20% for the lowest concentration and mean$\pm$15% for the other concentrations. All measured concentrations for the accuracy were within the range of $\pm$20% of the expected values.

CONCLUSION

Gas chromatography with nitrogen specific detector is the best current option for the analysis of diethylcarbamazine which lacks a chromophore with significant UV absorption. A standard curve of DEC assay was linear in the specified range with appropriate sensitivity for pharmacokinetic studies. The determination of diethylcarbamazine in plasma is easier and more reliable using solid phase extraction followed by GC-NPD. Solid phase extraction for sample preparation of DEC assay provided advantages of reducing labor, time of preparation, and lower use of organic solvent and glassware. One difficulty in using GC analysis of DEC in plasma for pharmacokinetic studies is the presence of the DEC-*N*-oxide metabolite, since it is thermally labile and is converted back to DEC or a material which coelutes with DEC under the conditions of the analysis. By separating DEC-*N*-oxide from DEC, there is no carry-over of DEC-*N*-oxide in the DEC analysis. In addition, solid phase extraction results in high reproducibility, accuracy and recovery. For pharmacokinetic studies, it is believed that the methods described above provide high specificity and greater sensitivity for DEC analysis in plasma.

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