IN VIVO METABOLISM OF THE ANTITUMOR IMIDAZOACRIDINONE C1311 IN THE MOUSE AND IN VITRO COMPARISON WITH HUMANS

C. R. CALABRESE, P. M. LOADMAN, L. S. E. LIM, M. C. BIBBY, 1,2 J. A. DOUBLE,1,2 J. E. BROWN,2 AND J. H. LAMB

Clinical Oncology Unit (C.R.C., P.M.L., L.S.E.L., M.C.B., J.A.D.) and Pharmaceutical Chemistry (J.E.B.), University of Bradford, Bradford, United Kingdom; and Medical Research Council Toxicology Unit (J.H.L.), Leicester, United Kingdom

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ABSTRACT:

C1311 has emerged as the lead compound from a novel group of anticancer agents, the imidazoacridinones, and will be entering clinical trials shortly. Previous murine pharmacokinetic studies have shown C1311 to be rapidly and extensively distributed into tissues including tumor. This study has identified two major metabolites of C1311 and describes their pharmacokinetics in mice. M1 is a glucuronide of the parent compound with high concentrations in both plasma and liver. Calculated area under the plasma concentration versus time curve values were 6-fold and 2-fold greater, respectively, than C1311. Based on these studies, we propose M2 to be a nonfluorescent oxidation product because electrospray ionization–mass spectrometry/mass spectrometry analysis gave a molecular ion at m/z 367, 16 U greater than the parent compound. It formed rapidly in liver preparations in vitro, both murine and human, by a cytosolic process in the presence of NADPH and in vivo was detected in liver tissues at concentrations equivalent to those of C1311 but was not detectable in plasma. Preliminary in vitro toxicity studies showed M2 to be as potent as C1311 against MAC15A tumor cells. Over the first 24 h, 39% of the administered dose is eliminated via the bile (28%) mostly as C1311 or the kidneys (11%) as the glucuronide (M1). This study has given valuable information as to the likely metabolic pathway to occur in humans, and the cytotoxic metabolite M2 may play a role in the antitumor activity or toxicity of C1311 in the clinic.

Imidazoacridinones are a novel group of anticancer agents with a design based on studies with mitoxantrone (Fig. 1a) (Cholody et al., 1990). The major features of the imidazoacridinones are a planar, polycyclic nucleus (capable of DNA intercalation) and a polyethylenediamine side chain. In addition, attachment of an imidazole ring to the chromophore was performed to increase the electron density of the π system, making the chromophore more resistant to enzymatic reduction to radical species, which was the rationale behind the development of anthrapyrazoles such as losoxantrone (Fig. 1b). In addition, the presence of the hydroxyl group at position 8 of the acridinone moiety appeared to be very important in the growth-inhibiting activity of the imidazoacridinones (Kusnierczyk et al., 1994), with metabolic activation within the cell postulated (Mazerska et al., 1996). C1311 (Fig. 1c) emerged as the lead compound from this group of agents and has been studied extensively both in vitro and in vivo against a range of colon tumors (both murine and human) (Burger et al., 1996). Similarities in the structures of C1311, mitoxantrone, and losoxantrone (CI-941) may suggest common cellular targets, with recent studies showing DNA intercalation (Burger et al., 1996) and topoisomerase II inhibition (Skladanowski et al., 1996). Resistance studies with C1311 versus a series of P-glycoprotein expressing human carcinoma multidrug resistance (MDR) cell lines (Warr et al., 1997) showed very little cross-resistance with both doxorubicin and the anthrapyrazole losoxantrone, supporting the lack of influence of P-glycoprotein in mechanisms of C1311 resistance. This lack of doxorubicin cross-resistance was also observed in MDR phenotype Friend erythro-leukemia cell lines (F4–6) (Berger et al., 1996), which confirmed that C1311 is only a weak substrate for P-glycoprotein. Interestingly, the imidazoacridinones were shown to be equally cytotoxic to both monolayer cultures and three-dimensional spheroids compared with mitoxantrone and doxorubicin, which show greater activity against monolayer cultures (Skladanowski et al., 1996), suggesting both good tissue penetration of C1311 and possible activity against the plateau phase or nondividing cells.

Preclinical pharmacokinetic studies for C1311 in mice have shown rapid and extensive distribution of drug into tissues, including liver, kidney, spleen, and small intestine, and large tissue/plasma ratios. Distribution was also observed in tumor tissue (Calabrese et al., 1998), with drug concentrations consistently higher than concentrations needed for in vitro activity. These high levels of drug seen in tumor tissue help explain the good antitumor activity of the compound. Pharmacokinetic studies in mice have shown linearity of kinetics with respect to dose between 15 and 100 mg·kg⁻¹ after i.p. administration, with disproportionate increases observed up to the maximum tolerated dose by this route (150 mg·kg⁻¹).

These preclinical studies have shown C1311 to have clinical po-
Materials and Methods

Chemicals and Reagents. C1311 and C1310 (used as an internal standard) were kindly supplied by Professor J. Konopa (Technical University of Gdańsk, Poland). All solvents were of high-performance liquid chromatography (HPLC) grade purchased from Fisher Scientific (Loughborough, UK), and all chemicals were purchased from Sigma (Poole, UK).

Chromatography. C1311 and metabolites initially were extracted and analyzed by HPLC using the method of Calabrese and Loadman (1997). Briefly, this consisted of a Spherisorb S5 ODS-1 (5 µm, 250 x 4.6 mm i.d.) C18 end-capped cartridge (Phase Separations, Deeside, UK) with a LiChro-spher 100 RP-18 (5-µm) guard column (Merck, Lutterworth, UK). A flow rate of 1.5 ml/min was maintained using two Waters 510 pumps, and a sample injection was made using a Waters 717 autosampler (Waters, Watford, UK). Detection was performed using a Merck F1050 fluorescence detector (using excitation and emission wavelengths of 420 and 520 nm, respectively) and/or a Waters 996 photodiode array (PDA) detector, which were connected in series with the chromatographic system. Data from both fluorescence and PDA detectors were processed using Waters Millennium software on a Digital premier PC. The mobile phase consisted of a disodium orthophosphate/citrate buffer [pH 4.0 containing 0.07% (v/v) triethylamine] combined with acetonitrile (50%). This mobile phase was used later to develop a gradient in which mobile phase A contained 5% acetonitrile and mobile phase B contained 60%. The gradient was run for 20 min at 20% B and then increased to 44% B between 20 and 60 min.

Metabolite Analysis in Mouse Urine. All animal experiments were carried out under a project license approved by the home office (London, UK) and UK Coordinating Committee on Cancer Research guidelines (Workman et al., 1998) were followed throughout. Noval Medical Research Institute (NMRI) male (n = 4) and female (n = 2) mice were treated with C1311 (50 mg/kg i.p.) and placed into a metabowl apparatus (Harvard Apparatus Ltd., Kent, UK) for a period of 24 h. The amount of urine and feces collected was quantified by weight. Fecal pellets were homogenized 1:4 in saline, and the homogenate was diluted further 1:5 with saline before analysis. Urine was diluted 1:10 in saline. Aliquots of each (100 µl) were removed and extracted using acetonitrile precipitation as described previously (Calabrese and Loadman, 1997) and analyzed using HPLC. Drug-free samples from control mice also were analyzed to assess potential interference from endogenous compounds. Calibration curves were produced over the range of 0.01 to 10 µg/ml by spiking drug-free samples with known quantities of C1311 and internal standard. These curves have been shown previously to be linear over this range with good reproducibility (<10% variation) (Calabrese and Loadman, 1997). Concentrations of metabolites and C1311 were calculated from C1311 calibration curves and adjusted for dilutions made. These concentrations then were converted to weight of compound per fraction and percentages were calculated. In addition, the percentage of dose observed in each fraction was calculated following the assumption that two mice (with a net weight of 50 g) would have been treated with 2.5 mg C1311 (for a 50 mg/kg dose).

Metabolite Analysis in In Vitro Liver Preparations. Microsomal preparations were prepared as described by Dolfini et al. (1973). Livers were excised from non-tumor-bearing NMRI male mice and placed in ice-cold phosphate-buffered saline (PBS). These livers were then weighed and homogenized 1:4 in PBS. The liver homogenate was centrifuged at 9360 g for 20 min using a Beckman Optima TL ultracentrifuge equipped with a Beckman TLA 100.4 rotor. The supernatant (S9 fraction) was removed and further centrifuged for 60 min at 104,000 g. After this second centrifugation, the supernatant (cytosol) was removed and the pellet was washed and recentrifuged before being resuspended in PBS to produce a final suspension containing the microsomes from 1 g of liver per 1 ml of PBS. Both cytosolic and microsomal preparations were kept on ice until required. Aliquots (1 ml) of either cytosol or microsomal liver fractions were spiked with C1311 to a final concentration of 100 µg/ml. The cofactor NADPH (Sigma) (2%, w/v, in saline) was then added to give a final 0.2% solution. Samples then were incubated for 4 h at 37°C with aliquots (50 µl) removed for analysis at 0 and 4 h. Controls were prepared under the same conditions but either NADPH, enzyme fraction, or C1311 solution was replaced with saline. All samples and controls were extracted as described previously before they were injected into the isocratic HPLC system. Cytosolic
and microsomal fractions from a human liver sample (IIAM, Leicester, UK) were prepared as for murine samples and analyzed by HPLC at 0, 3, and 20 h.

**Isolation of Metabolites.** Two predominant metabolites were detected in these studies and designated M1, which was detected in urine, and M2, which was detected in both murine and human cytosolic incubations. To isolate these two metabolites, urine or cytosolic incubations were extracted and injected onto the HPLC system using the gradient system described and metabolites were collected using a fraction collector (Waters). These fractions were subjected to centrifugal evaporation using a Jouans RC10.10 centrifuge connected to a RC90 cooled vacuum system (Jouans) to remove organic solvent. Aliquots of the remaining aqueous phase (200 µl) were then applied to 100 µg Phenyl (PH) Bond-Elut cartridges (Varian) that had been preconditioned with 1 ml each of methanol and distilled water. The samples were drawn through under vacuum on a VacElut SPS 24 vacuum system (Varian). Cartridges were then washed with 1 ml of triple distilled water and 2 ml of methanol applied to elute the metabolites. The purity and identity of the isolated metabolites were confirmed by HPLC and PDA-derived spectral comparison with original spectra from urine or cytosolic samples.

**Identification of Metabolites: β-Glucuronidase Incubation.** It was thought to be likely that M1, the most polar of the two metabolites, could be a glucuronide of C1311. Therefore, aliquots of purified metabolite (100 µl) were added to 1 ml of β-glucuronidase solution (sulfatase-free) and incubated for 30 min at 37°C. The bovine β-glucuronidase (Sigma) was made up in potassium phosphate buffer (75 mM, pH 6.8) containing 1% (w/v) bovine serum albumin (Sigma) to give an activity of 650 U/ml buffer. In addition, saline controls containing metabolite were incubated similarly. Samples (50 µl) were taken from each tube at 0 and 30 min and extracted as described previously. Extracted samples (60 µl) were injected onto the isotropic HPLC system and monitored using both fluorescence and PDA detection. The percentage of conversion of M1 to parent compound was calculated by assuming the saline control at the relevant concentration of M1 and time point to be 100%. The identity of the assumed C1311 peak resulting from incubation was confirmed by comparison of the PDA spectrum with standard C1311.

**Electrospray Ionization–Mass Spectroscopy/Mass Spectroscopy (ESI-MS/MS) Analysis.** ESI-MS/MS analyses were performed in positive ion mode (Jabbar et al., 1989). Cell culture conditions and details of the assay have been described previously (Phillips et al., 1992), but cells were exposed for 96 h to both C1311 and M2 at a range of concentrations of 0.001 to 10 µM. Results were expressed as percentage of survival assuming the untreated cells to represent 100% survival. IC_{50} values were calculated as the concentration of drug required to reduce cell number by 50%.

**Pharmacokinetics of C1311, M1, and M2 in Murine Plasma and Liver.** After treatment with C1311 (100 mg·kg⁻¹ i.p.), mice (female NMRI) were anesthetized with diethyl ether before blood samples were obtained by cardiac puncture at various time points (three mice per point) after drug administration. Blood was placed into heparinized tubes on ice. Livers were then excised as quickly as possible, sealed in foil, and placed into liquid nitrogen before storing at −20°C. Plasma was separated immediately by centrifugation (1000g for 15 min at 4°C) and stored at −20°C. Tissue and plasma extraction procedures and analytical methods have been described previously (Calabrese and Loadman, 1997). Before analysis, livers were thawed and then homogenized in mobile phase buffer at 4°C, and protein was precipitated using acetonitrile. Protein was also precipitated from the plasma samples using acetonitrile before analysis by HPLC using the gradient system. Concentrations of C1311 were calculated from calibration curves of C1311. Concentrations of M1 and M2 were also calculated from the same calibration curves as M1311 equivalents (i.e., the extinction coefficients for M1 and M2 were assumed to be the same as C1311). Pharmacokinetic parameters were calculated using standard pharmacokinetic calculations. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoidal rule, whereas the elimination rate constant (K_{el}) was calculated using log linear regression analysis of the terminal phase of the curve. Plasma half-lives were calculated from the equation: 0.693/K_{el} = T_{1/2}, T_{max} and C_{max} were taken directly from the curve.

**Results.** Chromatograms obtained from HPLC analysis of 24-h urine and feces samples showed the presence of two major peaks in both samples with retention times (RTs) of 19 and 56 min (Fig. 2). Comparison of PDA-derived spectra from these two compounds confirmed the peak at 56 min to be C1311 and that at 19 min (designated M1) to be spectrally similar to parent compound, implying that it was a metabolite of C1311 (Fig. 3). Much lower levels of at least eight other metabolites were also seen. All had spectra related to C1311 and were more polar than C1311. Non-drug-treated control urine showed no interfering peaks. Calculations of total drug eliminated show that 11% of total administered drug is eliminated in the urine. This mostly was composed of M1 (84%), with only low levels excreted as parent compound (16%). In feces the proportions were appreciably different from those in the urine, with 28% total administered drug eliminated via the bile. Drug eliminated via the bile was composed mostly of C1311 (86%), with low levels of M1 (5%) and M2 (9%) (M2 was not detected in the urine). Experiments with female mice gave similar results.

In vitro metabolism studies using the murine cytosolic fraction showed a decrease in C1311 concentration to 30% of controls after 4 h. This decrease in C1311 was accompanied by formation of a metabolite (designated M2) at 4.7 min (Fig. 4). Spectral analysis of this compound showed characteristics similar to C1311, although λ_{max} values were shifted (252–238, 280 –282, 374 –356, and 420 – 446 nm for C1311 and M2, respectively) (Fig. 3). This metabolite was not seen using fluorescence detection, and subsequent attempts using a range of excitation wavelengths did not indicate any fluorescent properties. Similar studies using microsomal incubations in the presence of NADPH showed a slight reduction in C1311 to 83% in relation to controls with far lower levels of the same metabolite, M2. Analysis of cytosolic samples isolated from human liver showed the formation of the same metabolite as in the murine study, which was confirmed by spectral analysis (Fig. 4). The human liver study also
confirmed that conversion of C1311 to M2 is predominantly a cytosolic process.

HPLC analysis of the isolated M1 metabolite showed the presence of minor contaminants; however, these represented only 0.7% of the M1 peak area. Spectral comparisons of isolated M1 with the original urine samples matched exactly. Incubation of purified M1 from treated NMRI mice with β-glucuronidase showed complete conversion of metabolite to parent compound after incubation for 30 min at 37°C. Controls without enzyme showed no conversion over the incubation time, which confirms metabolite M1 as a glucuronide conjugate of parent compound.

After the isolation of metabolite M2, HPLC analysis showed the preparation to be >97% pure, and peak identity was confirmed by comparison with original cytosolic incubations. The two electrospray product ion spectra obtained for the quasi-molecular ions of C1311 and M2 are shown in Fig. 5 ([M+H]+ at m/z 351 and 367, respectively). Both spectra have common product ions at m/z 100, which is likely to represent part of the polyethylenediamine side chain \([\text{CH}_2\text{CH}_2\text{N(CH}_2\text{CH}_3)_2]\)\(^+\). Two different product ions were seen at m/z 278 (for C1311) and m/z 294 (for metabolite M2), suggesting the increase in molecular mass of the metabolite (16 U) is related to the polycyclic nucleus.

Preliminary in vitro toxicity studies showed M2 to be as potent as C1311 against MAC15A cells, and typical IC\(_{50}\) values were in the range of 0.5 μM for both C1311 (0.16 ± 0.2 μM) and M2 (0.40 ± 0.17 μM).

Pharmacokinetic studies after i.p. treatment of female NMRI mice with C1311 (100 mg·kg\(^{-1}\)) showed that C1311, M1, and M2 were present in liver samples and only C1311 and M1 were detectable in plasma. Comparison of the liver pharmacokinetic profiles for the three analytes (Fig. 6) showed M1 and C1311 to exhibit very similar characteristics, although the elimination phase was lengthened for both M1 and M2 compared with C1311 (T\(_{1/2}\) values of 3.3, 8.1, and 6.4 h for C1311, M1, and M2, respectively) (Table 1). The formation of both M1 and M2 appeared quite rapid, with T\(_{\text{max}}\) values of 1 h for both metabolites, although M2 reached an appreciably lower C\(_{\text{max}}\) (161.3, 166.4, and 28.8 μg·g\(^{-1}\)) for C1311, M1, and M2, respectively). AUC values that were obtained showed a higher value for M1 (405 μg·h·g\(^{-1}\)) compared with C1311 and M2, which were relatively similar (238 and 289 μg·h·g\(^{-1}\)) (Table 1).

The pharmacokinetic profiles observed for C1311 and M1 in plasma (Fig. 7) showed rapid distribution of M1 into plasma, with a T\(_{\text{max}}\) of 1 h (Table 2). The elimination T\(_{1/2}\) values showed the characteristic lengthy elimination for C1311 observed in all i.p. studies (11.95 h), with more rapid elimination of M1 (3.35 h). The AUC value obtained for M1 was approximately 6-fold larger than that observed for the parent compound (58.4 and 9.64 μg·h·ml\(^{-1}\)) (Table 2).

Discussion

C1311 is a member of a new group of compounds for which the pharmacokinetic characteristics of the parent compound have been described (Calabrese et al., 1998), but there is no information on its metabolism. The aim of this study, therefore, was to investigate the metabolism and elimination of C1311 in mice to and give insight into the
possible metabolic pathway in humans before its clinical application. We have described here two major metabolites of C1311, both of which are likely to be found in humans. Metabolite M1 has been shown to be the glucuronide, presumably with the conjugation occurring through the hydroxyl group at position 8 of the acridinone moiety. High concentrations of M1 were found in both plasma and liver of mice, and calculated AUC values were 6-fold and 2-fold greater, respectively, than C1311. Although the cytotoxicity of M1 was not assessed, glucuronides generally are far less potent than the parent compound but can contribute indirectly by enabling enterohepatic recycling of the compound to occur, thereby influencing the pharmacokinetics of the parent compound. The glucuronide was not detected in the feces, but it is quite feasible that the high levels of C1311 found in the feces were a result of hydrolysis of the glucuronide in the intestine. Conjugation of drugs by glucuronyl transferases plays an important role in drug disposition (Kroemer and Klotz, 1992), and, so, any species differences that may exist in glucuronyl transferases expression could have a large influence on the pharmacokinetics of the parent compound.

M2 is produced rapidly in vitro by both murine and human liver preparations predominantly by cytosolic enzymes in the presence of

\[ \text{TABLE 1} \]

<table>
<thead>
<tr>
<th>Compound</th>
<th>AUC ((\mu g \cdot h \cdot g^{-1}))</th>
<th>(T_{1/2}) (h)</th>
<th>(K_{el}) (h)</th>
<th>(T_{max}) (h)</th>
<th>(C_{max}) ((\mu g \cdot g^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>C1311</td>
<td>238</td>
<td>3.3</td>
<td>0.21</td>
<td>0.25</td>
<td>161.3</td>
</tr>
<tr>
<td>M1</td>
<td>405</td>
<td>8.1</td>
<td>0.086</td>
<td>1</td>
<td>166.4</td>
</tr>
<tr>
<td>M2</td>
<td>289</td>
<td>6.4</td>
<td>0.11</td>
<td>1</td>
<td>28.8</td>
</tr>
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</table>

Fig. 5. Electrospray ion spectra obtained for C1311 (a) and M2 (b) giving a molecular ion \((M+H)^+\) of 351 and 367, respectively. Both spectra have common ions at m/z 100, which is likely to represent part of the polyethylenediamine side chain \([\text{CH}_2\text{CH}_2\text{N} (\text{CH}_2\text{CH}_3)_2]^+\).

Fig. 6. Concentration of C1311, M1, and M2 in murine liver after administration of C1311 (100 mg \( \cdot \) kg\(^{-1}\)).
the formation of a mitoxantrone-glucuronide conjugate with rat liver fractions although the conjugate was not seen in human urine. Biliary excretion of mitoxantrone predominates in both rats and humans (Alberts et al., 1985; Batra et al., 1986), and a further metabolite was identified by Blanz et al. (1991), although this was a result of the enzymatic oxidation of the phenylenediamine side chain of mitoxantrone and, therefore, is not likely to be relevant to the metabolites described in this article.

In summary, this study has identified two major metabolites of C1311: a glucuronide and a cytotoxic oxidation product, both of which are formed rapidly in the liver and retained at relatively high concentrations for a long period of time. Over the first 24 h, approximately 28% of the administered dose was eliminated via the bile mostly as the parent compound and 11% of the dose was eliminated via the kidneys as the glucuronide. The cytotoxic oxidation product (M2) also has been shown to be formed in human liver preparations. This study has provided valuable information on the metabolic pathway likely to occur in humans, and this may be of significant value in interpreting the results of the phase I trials. Identification of M2 as a cytotoxic metabolite is important because this metabolite may have a role to play in its antitumor activity or toxicity.

**References**


**TABLE 2**

<table>
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<tr>
<th>Compound</th>
<th>AUC</th>
<th>$T_{1/2}$</th>
<th>$K_{el}$</th>
<th>$T_{max}$</th>
<th>$C_{max}$</th>
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<tr>
<td>C1311</td>
<td>9.64</td>
<td>11.9</td>
<td>0.058</td>
<td>0.083</td>
<td>11.4</td>
</tr>
<tr>
<td>M1</td>
<td>58.4</td>
<td>3.3</td>
<td>0.207</td>
<td>1</td>
<td>13.0</td>
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</table>

**Figure 7.** Concentration of C1311 and M1 in plasma after administration of C1311 (100 mg·kg$^{-1}$ i.p.).

Metabolite M2 was not detected in plasma.

NADPH. It could be detected in liver tissues at concentrations equivalent to those of C1311 but was not detectable in plasma. It may well be conjugated and excreted rapidly in the bile, although we saw no evidence of an M2 conjugate in the feces—only low levels of M2 alone. It would appear to be a cytotoxic metabolite of C1311, as shown by in vitro studies demonstrating equal potency to C1311, and presumably would have a similar mechanism of action capable of intercalation and topoisomerase II inhibition.

LC-ESI-MS analysis of purified M2 showed a molecular ion at m/z 367, 16 U greater than the parent compound. This probably arises via the addition of a single atom of oxygen and is consistent with 10-hydroxylation in ring A to produce a catechol system, which may undergo further oxidation to produce the corresponding ortho-quinone nonfluorescent species. However, because the metabolism of C1311 to M2 is predominantly a cytosolic process, a more likely explanation is that a nonmixed function is oxidase-based oxidation of the benzimidazol-2-one ring system. This also would be consistent with the observed loss of fluorescence seen with M2 because the benzimidazol-2-one ring system will not contribute to the conjugation of the antraphenyleneimine system, unlike the parent compound. This lack of fluorescence also means the sensitive fluorescent-detection method used for C1311 cannot be used for M2, although detection at 238 or 446 nm was adequate but not as sensitive.

Comparisons with the metabolism of related compounds is difficult because both mitoxantrone and losoxantrone are metabolized predominantly through their hydroxy ethylamino ethylamino side chains, with the major human metabolites being the mono- and dicarboxylic acid derivatives (Chiccarelli et al., 1986). Macpherson et al. (1984) have described