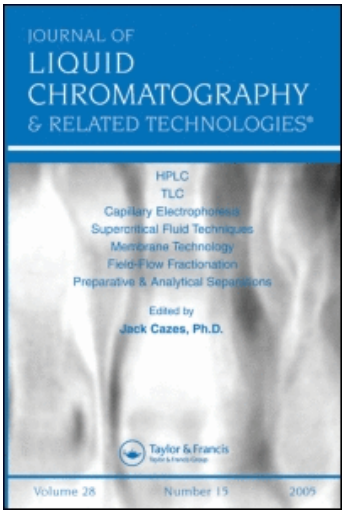

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HPLC Analysis of Merbarone (NSC 336628) in Plasma Using MG(II) Asamobile Phase Modifier

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HPLC ANALYSIS OF MERBARONE (NSC 336628) IN PLASMA USING MG(II) AS A MOBILE PHASE MODIFIER

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ABSTRACT

Merbarone is a thiobarbituric acid derivative which has been shown to be curative in murine leukemias and is currently under evaluation in the treatment of cancer patients. A reversed-phase HPLC method was developed for the quantitation of plasma levels of merbarone. The protein of plasma (50 μl) was precipitated with 250 μl of methanol-DMSO (82:18, v/v) containing the internal standard, 3'-F-merbarone. After centrifugation, the supernatant (200 μl) was diluted with the mobile phase less methanol (300 μl) and 350 μl was injected on column. Chromatography was carried out on a 4 μm Nova-Pak C_{18} column (3.9 mm x 15 cm) using an isocratic mobile phase and UV detection at 306 nm. The mobile phase was methanol-ammonium acetate (0.1 M)-acetic acid (0.4 M)-$MgSO_4 \cdot 7H_2O$ (2.08 M) (30:60:8:2, by volume) containing 1 mM sodium dodecyl sulfate. Employing 50 μl of plasma, concentrations of merbarone in the range 0.05-5.00 μg/ml were quantitated. Very low levels of one metabolite, tentatively identified as 2-oxo-desthiomerbarone, were observed in chromatograms of patient plasma.

INTRODUCTION

Merbarone (NSC 336628), 5-(N-phenylcarboxamido)-2-thiobarbituric acid (Figure 1), was chosen for evaluation clinically as a chemotherapeutic agent when it was shown to exhibit curative activity against the murine P388 and L1210 leukemias in addition to good antitumor activity against solid tumors, the B16 melanoma and M5076 sarcoma (1-3). During the phase I clinical trials at this institution, the analytical method

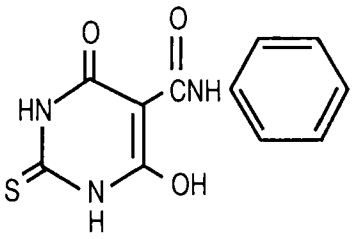

FIGURE 1. Chemical structure of merbarone.

described in this paper was used to monitor the pharmacokinetics of merbarone administered as a 120 hour continuous intravenous infusion (civ) and as a daily 2 hour civ for 5 days. Doses were escalated from 96-1250 mg/m²/day x 5 in the phase I study of the 120 hour schedule and from 150-447 mg/m²/d x 5 for the 2 hour regimen. Currently, the compound is undergoing evaluation in phase II clinical trials in solid tumors.

Eluents containing metal ions without ligands to facilitate the determination of organic compounds which form complexes have been used infrequently in liquid chromatography (4-7). In this work, we describe an HPLC assay for merbarone in plasma employing a mobile phase containing Mg(II). Plasma protein precipitation with methanol-DMSO (82:18, v/v) was followed by injection of the supernatant onto the chromatographic column. Chromatography was carried out under isocratic conditions and the drug was monitored by UV detection at 306 nm. A structural analog of the drug, 3'-F-merbarone, served as the internal standard. Employing 50 μl of plasma, the lowest concentration of the standard curve was 0.050 μg/ml.

The present assay was derived from one which we previously developed for characterizing the preclinical pharmacology of merbarone in mice and Beagle dogs (1,8). The former method was also isocratic, employing methanol-ammonium acetate (0.5 M)-magnesium chloride (1 g/ml) (25:75:2, v/v/v) as the mobile phase and the same merbarone analog as internal standard. This assay was provided to the National Cancer Institute and used for monitoring plasma levels during toxicology studies of the civ regimen to Beagle dogs (9). It was reported after modification as a gradient method (10).

Prior to the use of the method for patient pharmacokinetic studies, two modifications of our previous method were sought. The first inadequacy, corrosion of

the stainless steel tubing and fittings in the chromatographic system, was resolved by the replacement of magnesium chloride with magnesium sulfate. Secondly, the analytical columns which were used for the assay of dog and mouse plasma had provided a short period of service, despite the use of inline filters and a guard column. This difficulty was resolved by the addition of acetic acid and a surfactant to the mobile phase. Our earlier procedure for plasma sample preparation was unchanged.

MATERIALS AND METHODS

Reagents and Chemicals

Merbarone (NSC 336628, lot 058-55 sample NB), the internal standard, 3'-F-merbarone (NSC 372106) and a reference sample of 2-oxo-desthiomerbarone (NSC 366236), were obtained from the Pharmaceutical Resources Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The purity of merbarone was 99.4% based upon the provided spectral and liquid chromatographic data.

The ammonium acetate and acetic acid were analyzed reagents (J.T. Baker Chemical Co., Phillipsburg, NJ). Reagent grade magnesium sulfate heptahydrate was used (Mallinkrodt, Paris, KY). The methanol was glass distilled (OmniSolv grade, E.M. Science, Cherry Hill, NJ) and the dimethylsulfoxide (DMSO) was HPLC grade from Burdick and Jackson Laboratories (Muskegon, MI). Dodecyl sodium sulfate was purchased from Eastman Kodak Co. (Rochester, NY). Double distilled deionized water was filtered through a 0.2 μm Nylon-66 filter (Rainin Instrument Co., Woburn, MA) before use.

In the complexation studies, monobasic potassium phosphate was a Baker analyzed reagent (J.T. Baker Chemical Co.); dibasic potassium phosphate and potassium chloride were analytical reagents (Mallinckrodt). Reagent grade magnesium chloride hexahydrate (E.M. Science), magnesium sulfate heptahydrate and potassium hydroxide (Fisher Scientific, Fair Lawn, NJ) were used. All chemicals were used without additional purification. The deionized double distilled water was boiled before use.

Apparatus

Chromatography was performed using a model 114M pump (Beckman Instruments, Berkeley, CA), a WISP 712 automatic injector (Waters Associates, Milford,

MA) fitted with a 2000 μl loop and a variable wavelength Spectroflow model 783 programmable absorbance detector (ABI Analytical, Kratos Division, Ramsey, NJ) containing a 12 μl flow cell (pathlength 8 mm). The 1 volt output of the detector was provided as the signal to a model 3393A recording integrator (Hewlett-Packard, Avondale, PA). The system was equipped with a stainless steel 3.9 mm x 15 cm column packed with 4 μm Nova-Pak C_{18} (Waters Associates). A 0.5 μm filter (Rainin Instrument Co.) and a Guard-Pak precolumn containing a 10 μm μBondapak C_{18} insert (Waters Associates) was installed before the analytical column.

UV spectra were obtained during chromatography with a second analytical system configured similarly but with a Hewlett-Packard (Palo Alto, CA) model 1040M diode array detector and a model 7125 manual injector (Rheodyne, Cotati, CA) equipped with a 200 μl loop. Absorption spectra during complexation studies were recorded with a Beckman model 3600 UV-VIS Spectrophotometer (Beckman Instruments, Irvine, CA) using matched 1.0 cm quartz cuvettes.

Milligram quantities of the samples used for preparing stock solutions were weighed on a Cahn 25 electrobalance (Cahn Instruments, Inc., Ceritos, CA). Stock solutions were prepared in class A borosilicate glass volumetric flasks. All glassware used in the plasma assay was treated with a siliconizing reagent (Surfasil in toluene, Pierce Chemical Co., Rockford, IL). Glassware used in complexation studies was soaked in aqueous sulfuric acid, then thoroughly rinsed with distilled water and oven dried. An Eppendorf model 5412 microcentrifuge (Brinkmann Instruments, Westbury, NY) was used for centrifuging assay samples contained in 1.5 ml polypropylene microcentrifuge tubes (VWR Scientific Inc., Philadelphia, PA) at 12,000 x g.

Solutions in Complexation Studies

A pH 7.0 phosphate buffer, ionic strength 0.01, was made from 14.36 ml of 0.02 M KH_2PO_4 and 23.94 ml of 0.01 M K_2HPO_4 and diluting to 100 ml with water. Stock solutions of merbarone and $MgSO_4$ were prepared in the buffer to afford concentrations of 1.0×10^{-4} M and 6.3×10^{-2} M, respectively. The merbarone solution was prepared by dissolving 1.374 mg in 10.0 ml of 0.001 N KOH and diluting to 50 ml with buffer. Lower concentration $MgSO_4$ solutions were obtained by 10, 100 and 1000-fold serial dilutions with buffer.

A series of solutions containing a constant merbarone concentration (2.1×10^{-5} M) with Mg(II):merbarone molar ratios of 0-1000 were prepared by adding 3.0 ml of merbarone stock solution and 5.0 ml of aqueous $MgSO_4$ ($0.0\text{-}6.3 \times 10^{-5}$ M) to phosphate

buffer (7.0 ml). After magnetically stirring for 1 min, an aliquot (3 ml) of the solution was immediately transferred to the sample cuvette and its absorption spectrum was scanned from 350-200 nm at 100 nm/min utilizing a normal slit program and 2 second period against a reference of phosphate buffer. Spectral measurements were conducted at ambient temperature. Samples were visually inspected for the presence of turbidity before and after scanning.

Protein Precipitant Solution

The internal standard stock solution was 1.05 mg/ml of 3'-F-merbarone in DMSO. After dilution to 10.5 μg/ml in DMSO, a 2.4 ml aliquot was added to 60 ml of methanol-DMSO (85:15, v/v) to provide the solution used in the assay for protein precipitation and drug isolation.

Plasma Standards

Stock solutions of merbarone in DMSO were prepared to provide a concentration of approximately 0.5 mg/ml. Plasma standards with a range of concentrations from 0.049-4.91 μg/ml were prepared. The addition of varying volumes of the merbarone stock solution to drug-free plasma in siliconized glass test tubes yielded solutions containing 1.98, 2.96, 3.94 and 4.91 μg/ml. Serial dilution of the 1.98 μg/ml standard with drug-free plasma afforded five additional solutions ranging in concentration from 0.049-0.99 μg/ml. These solutions were thoroughly mixed by vortexing for 1 min.

Sample Preparation

To 50 μl of plasma in a microcentrifuge tube was added 250 μl of the protein precipitant solution containing the internal standard. The tube was stirred by vortexing for 0.5 min and then centrifuged for 10 min. An aliquot of the supernatant (200 μl) was separated from the precipitated protein and diluted with 300 μl of the mobile phase less methanol in a microcentrifuge tube. The solution was mixed by vortexing and transferred to a polypropylene insert, which was placed in the automatic sampler. The injection volume was 350 μl. For plasma samples containing concentrations exceeding the range of the standard curve, an aliquot of the sample was diluted with drug-free plasma to make the total volume 50 μl.

Chromatographic Conditions

The mobile phase consisted of methanol-ammonium acetate (0.1 M)-acetic acid (0.4 M)-$MgSO_4 \cdot 7H_2O$ (2.08 M) (30:60:8:2, by volume) containing 1 mM sodium dodecyl sulfate. For various analytical columns, the fraction of the methanol was adjusted, typically between 25-30% of the mobile phase volume, to minimize variation in the retention times of the drug and internal standard. The solution was degassed in an ultrasonic bath for 15 min prior to chromatography.

Chromatography was performed at ambient temperature with a flow rate of 1.0 ml/min. Absorbance of the column effluent was monitored at a wavelength of 306 nm. The integrator was configured to report peak areas employing a 0.3 min peak width and a threshold setting of 0. Chromatograms were recorded with a 0.2 cm/min chart speed and an integrator attentuation of 2.

Quantitation

Standard curves, which were prepared daily, were constructed by plotting the peak area ratios of merbarone to the internal standard against the merbarone concentration. The best fit straight line was determined by least squares regression using a weighting factor of reciprocal peak area ratio squared to calculate the slope, y-intercept and correlation coefficient. Merbarone concentrations in unknown samples were calculated using the results of the regression analysis.

Absolute Recovery

The absolute recovery of merbarone was carried out by diluting the DMSO stock solution of merbarone with methanol-DMSO (85:15, v/v) to provide concentrations which were the same as the plasma standards carried through the precipitation procedure. Similar injection volumes were used for both sets of samples. The ratio of merbarone peak area from the precipitated plasma standards to that for corresponding direct-injection samples gave a measure of the absolute recovery. A similar procedure employing a single concentration of the internal standard was utilized.

Relative Recovery

The relative recovery of merbarone was computed from the calculated concentrations of the compound in plasma standards assayed during a four week period.

RESULTS

Plasma Sample Preparation

Plasma samples containing merbarone were deproteinized by precipitation with methanol-DMSO (82:18, v/v). After diluting the supernatant with mobile phase less methanol to adjust the solvent strength, the sample was chromatographed using a reversed-phase system containing magnesium ion with detection of the drug by UV absorption at 306 nm. The duration of chromatography for a single sample was 20 min, permitting a calibration curve, consisting of 9 plasma standards plus a drug-free sample, and the samples of a pharmacokinetic profile to be analyzed in a working day.

Liquid Chromatography

The very high adsorption of merbarone to reversed-phase column packings was demonstrated by pronounced tailing of chromatographic peaks for both the drug and internal standard. Severe tailing occurred on various stationary phases (ODS, octyl, cyano and phenyl) with eluents composed of methanol or acetonitrile and acetate or phosphate buffers in the range pH 4-7. Broad, tailed peaks also occurred when using alkaline mobile phases and a PRP-1 column (Hamilton Co., Reno, NV). Adsorption was reduced in the presence of a high ammonium acetate concentration, but not sufficiently.

Incorporating divalent magnesium into an eluent containing ammonium acetate and methanol appeared to afford chelates of the drug and internal standard which chromatographed with a minimum of tailing. Of the various analytical reversed-phase columns examined for their ability to resolve merbarone and the internal standard using this mobile phase, optimal separation was achieved with the Nova-Pak C_{18} column. Different Nova-Pak C_{18} columns resulted in retention times for merbarone which varied from approximately 6.5-9.5 min and 11.0-14.5 min for the internal standard. Adjusting the amount of methanol in the mobile phase within the range 25-30% (v/v) afforded retention times which were reasonably consistent between columns.

Figure 2A shows a chromatogram of drug- and internal standard-free human plasma. The plasma samples generally afforded clean chromatograms without interfering endogenous peaks. Chromatograms determined at 306 nm of human plasma containing merbarone at concentrations of 0.049, 0.988 and 4.91 μg/ml are shown in Figure 2. Chromatograms of plasma obtained from a patient, prior, during and after administration of merbarone, 192 mg/m²/day by 120 hour civ, are shown in Figure

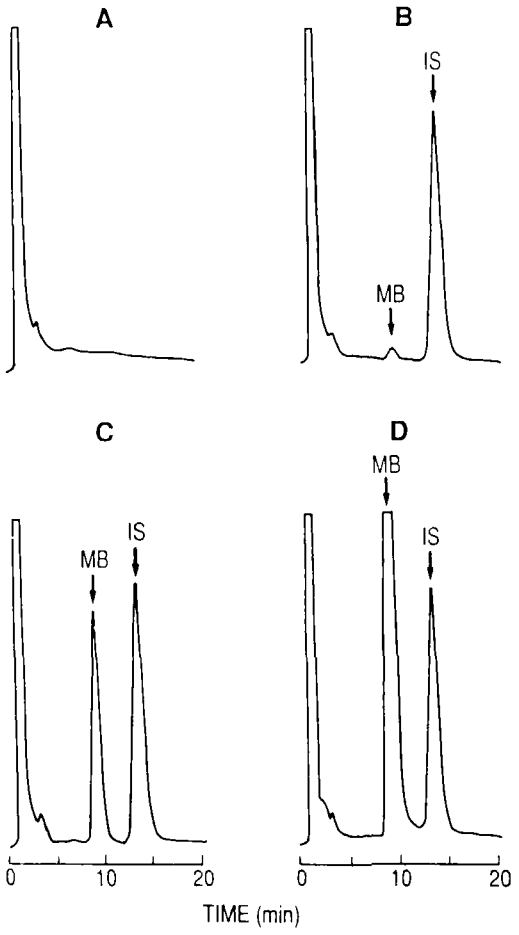

FIGURE 2. Liquid chromatograms of merbarone (MB) and internal standard (IS) added to human plasma. (A) Drug- and IS-free plasma; (B) merbarone, 0.0494 μg/ml; (C) merbarone, 0.9880 μg/ml; (D) merbarone, 4.911 μg/ml.

3. The pretreatment plasma (Figure 3A) showed no UV-absorbing impurities which would interfere with detection of either the drug or internal standard.

Although retention times of the drug and internal standard generally did not vary greatly (0.5 min) during each day, two principal factors contributed to the day-to-day variation. The analytical column was protected by a precolumn C_{18} cartridge, which was replaced each day, since deproteinated plasma without filtration was injected

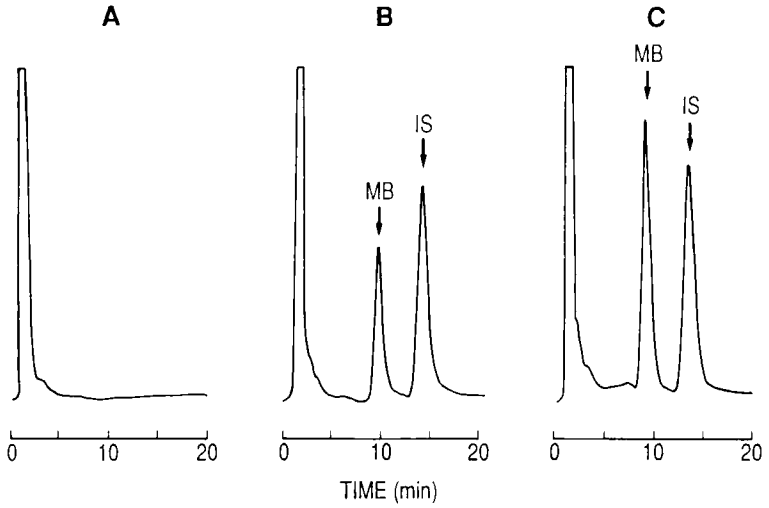

FIGURE 3 Liquid chromatograms of merbarone (MB) and internal standard (IS) in human plasma during and after civ of 192 mg/m²/day x 5. (A) Pre-treatment plasma; (B) merbarone, 4.24 μg/ml in a sample obtained at 3.14 hr; (C) merbarone, 6.40 μg/ml in a sample obtained at 144.13 hr.

directly. The capacity factors of both the drug and internal standard varied with the change of cartridge. In addition, each week the column was reversed and flushed, first with water (300 ml), then with methanol (300 ml) and finally with methanol-water (3:1, v/v). Subsequent to flushing, equilibration of the column with the mobile phase was reestablished by pumping at 1.0 ml/min overnight.

Calibration Curves

Figure 4 represents a typical calibration curve in which the merbarone to internal standard peak area ratio is proportionate to the concentration of merbarone. The best fit lines of the standard curves, encompassing a 40-fold range of concentrations, were obtained by linear regression analysis employing a weighting factor of the reciprocal peak area ratio squared. The correlation coefficients were generally greater than 0.998.

Absolute Recovery and Assay Reproducibility

The mean absolute recovery of merbarone from plasma (n = 5) with concentrations 0.050, 0.125, 0.998, and 4.96 μg/ml was 99.4 ± 7.1%, 97.3 ± 4.7%, 101.6

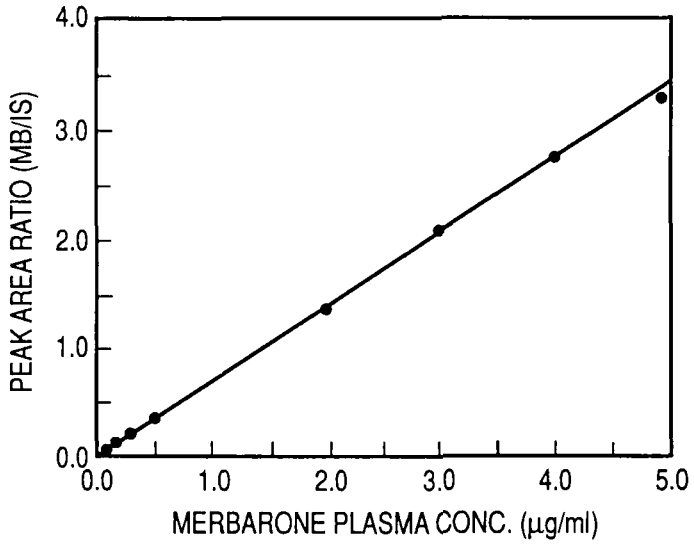

FIGURE 4 Calibration curve for merbarone in human plasma. Slope = 0.6911, intercept = 0.002154, r = 0.9997.

± 5.4, and 99.6 ± 2.7%, respectively. The mean absolute recovery (n = 20) of the internal standard was 95.5 ± 5.9% of the amount added to each plasma sample (0.101 µg).

Plasma standards were prepared on 9 days during a four week period from a single stock solution of merbarone. The relative recoveries of merbarone from plasma and the assay reproducibility for the range of concentrations of the standard curve are summarized in Table 1. Coefficients of variation for the replicate assays ranged from 1.12-4.84% indicating that the analytical method is reproducible. The recovery ranged from 98.9-101.2% of the amount of merbarone added to plasma samples. The coefficient of variation of the averaged slopes of the linear regression curves was 4.43%, demonstrating that the assay was consistent during this period.

Metabolites and Specificity of the Analytical Method

Very low levels of one metabolite, exhibiting the R_T of 2-oxo-desthiomerbarone, were observed in chromatograms of patient plasma. Chromatograms demonstrating assay specificity were determined with patient plasma obtained on day 5 during the 120 hour civ of 144 mg/m²/day doses of the drug. A three-dimensional chromatogram from

TABLE 1

Relative Recovery and Reproduciblity of the Analytical Method for Merbarone in Human Plasma[a]

Amount Added (μg/ml)	Mean Amount Found (μg/ml)	Recovery %	Coefficient of Variation %
0.0494	0.0497	100.6	2.41
0.1235	0.1239	100.3	4.84
0.2470	0.2476	100.2	2.79
0.4940	0.4918	99.6	1.12
0.9880	0.9768	98.9	2.32
1.976	1.979	100.2	2.28
2.958	2.994	101.2	2.01
3.937	3.981	101.1	1.41
4.911	4.879	99.3	2.33

[a] Number of replicates = 9.

1.4-18 min of pre-treatment plasma exhibited a small peak at about 3 min and was otherwise free of interfering components (Figure 5A). A similar chromatogram of plasma obtained on day 5 during dosing showed the parent drug at 8.4 min and only an exceedingly small amount of the metabolite at 6.9 min (Figure 5B). Enhanced visualization of the metabolite was realized by truncating the merbarone peak (Figure 5C).

The UV spectra acquired at the upslope, apex, and downslope of the merbarone peak in a chromatogram of patient plasma obtained on day 5 during the civ of 1000 mg/m^2/day were similar (Figure 6A). The spectrum determined at the peak apex was similar to that of a reference sample (Figure 6B). The levels of 2-oxo-desthiomerbarone present in the plasma of patients treated at this dose level permitted good examination of the metabolite spectrum. A satisfactory overlay was obtained of the UV spectra at the centroid of chromatographic peaks from a synthetic sample of the compound and the metabolite in human plasma (Figure 6C).

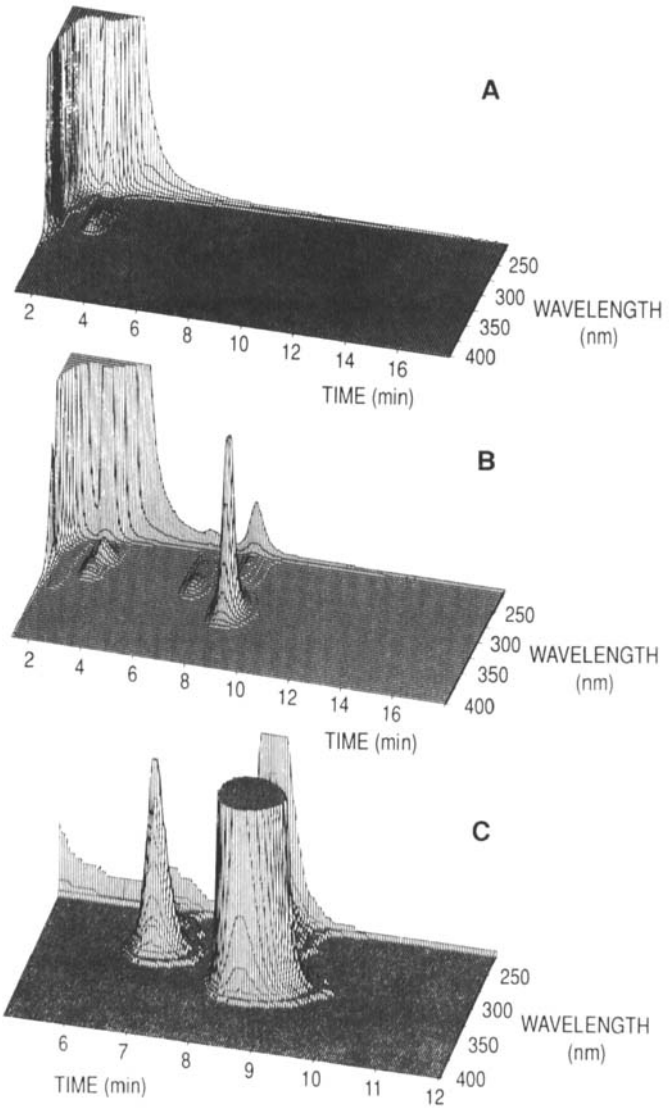

FIGURE 5 Three-dimensional chromatograms of patient plasma, pre-treatment and on day 5 during the civ of merbarone, 144 mg/m²/day x 5. (A) From 1.4 to 18 min of pre-treatment plasma showing the absence of constituents which would interfere with the detection of merbarone; (B) from 1.4 to 18 min of plasma obtained on day 5 illustrating the low level of metabolite; (C) from 5 to 12 min of plasma obtained on day 5, showing the merbarone peak truncated to permit inspection of the metabolite peak. Chromatographic peaks: (6.9 min) 2-oxo-desthiomerbarone, (8.4 min) merbarone.

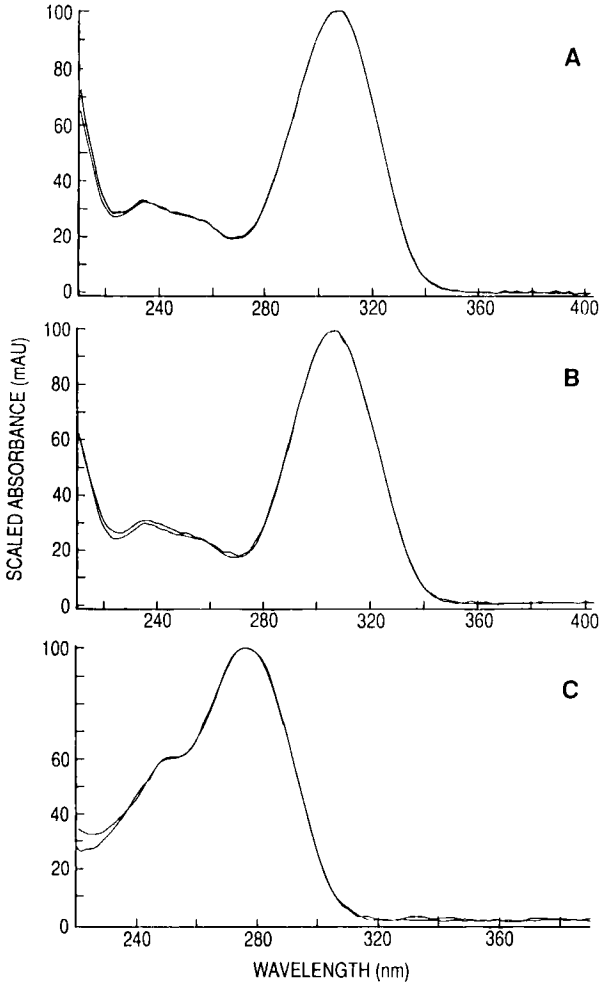

FIGURE 6 Overlay of UV spectra determined during chromatography of patient plasma obtained on day 5 during civ infusion infusion of 1000 mg/m²/day x 5 and reference standards. (A) At the upslope, apex, and downslope of the merbarone peak in plasma; (B) at the centroid of the chromatographic peaks of a reference sample and merbarone in plasma; (C) at the apex of the chromatographic peak of a reference sample of 2-oxo-desthiomerbarone and the metabolite in plasma.

TABLE 2

UV Absorbance Values at Selected Wavelengths for Varying Molar Ratios of Magnesium Sulfate to Merbarone in pH 7 Phosphate Buffer

$MgSO_4$ Conc. (M)	Molar Ratio Mg(II)/MB	λ_{max}	Absorbance A_{300}	A_{251}	A_{237}
0.0	0.00	303	0.575	0.280	0.255
2.102×10^{-5}	1.01	303	0.578	0.276	0.248
2.102×10^{-4}	10.05	304	0.579	0.262	0.244
2.102×10^{-3}	100.48	306	0.585	0.230	0.241
2.102×10^{-2}	1004.83	307	0.687	0.198	0.240

Complexation with Magnesium Ions

A summary of the spectral changes that occured as the Mg(II):merbarone molar ratio was increased while maintaining the same concentration of merbarone in pH 7 phosphate buffer are shown in Table 2. The presence of $MgSO_4$ produced a bathochromic shift and an increase in absorptivity in the λ_{max} which was 303 nm in the absence of Mg(II). Furthermore, the λ_{max} at 251 nm disappeared with concurrent development of a new peak at 237 nm as the molar ratio was increased. The same spectral perturbations were observed when $MgSO_4$ was replaced by $MgCl_2$. Neither magnesium salt absorbs in this region of the spectrum at the concentrations employed. Changes in the spectrum of merbarone were not apparent in the presence of KCl at concentrations similar to the magnesium salts. Accordingly, these spectral perturbations are a consequence of complexation between merbarone and Mg(II).

DISCUSSION

Physical Properties of Merbarone

The properties of merbarone which significantly affected the development of an analytical method were (a) limited solubility in many organic solvents and in water, (b) adsorption of the compound from aqueous solutions onto the surface of glass or plastic

containers, and (c) strong adsorption of the compound by all liquid chromatographic packings.

Merbarone has limited solubility (< 0.1 mg/ml) in many common organic solvents (chloroform, methylene chloride, benzene, methanol, ethanol, acetonitrile, acetone, trifluoroacetic acid) and aqueous solutions with pH less than 9. The compound is soluble in DMSO (≈ 80 mg/ml), tetrahydrofuran (≈ 6 mg/ml) and in strongly alkaline aqueous solutions (e.g., 0.1 N NaOH, ≈ 5 mg/ml). The limited solubility of merbarone in the solvents commonly employed for extraction precluded that conventional technique for isolating the drug from plasma. Recovery from plasma with solid phase extraction columns was low and highly variable, attributable to the high adsorptivity of merbarone. Accordingly, the development of a method based upon direct injection of plasma after protein precipitation was undertaken.

The solubility of merbarone in tetrahydrofuran had prompted its initial selection as a protein precipitant and as the solvent for the preparation of stock solutions of the drug. However, it was found that the concentration of drug in tetrahydrofuran decreased with time as a consequence of its oxidation to 2-oxo-desthiomerbarone induced by peroxides which formed in the solvent. Hydrogen peroxide has been shown to oxidize 2-thiobarbiturates to barbiturates (11). While no loss of compound occurred when the tetrahydrofuran was treated to remove peroxides, its use was discontinued.

A stock solution of merbarone in DMSO proved to be stable for at least three months. Losses due to the adsorption of the drug to siliconized glass or plastic surfaces did not occur from a solution in methanol-DMSO (82:18, v/v) and therefore, plasma protein precipitation was carried out with this solvent mixture. While adsorption of the compound to surfaces occurred from aqueous or hydroalcoholic solutions, adsorptive losses were not apparent from the plasma standards, undoubtedly due to a very high degree of plasma protein binding.

Liquid Chromatography

Liquid chromatography of merbarone on reversed-phase columns generally afforded broad peaks with extensive tailing. The development of a suitable liquid chromatographic method for merbarone in plasma was largely unsuccessful until magnesium ion was included as a component of the mobile phase. The premise was that complexation of merbarone may afford a species with decreased affinity toward adsorption. Inclusion of magnesium in the mobile phase, initially as magnesium chloride and in the present method by addition of magnesium sulfate, greatly improved the peak

shape of merbarone. Accordingly, studies were undertaken to determine whether complexes between merbarone and magnesium ion were formed. It is recognized that the presence of magnesium in the mobile phase may also modify characteristics of the stationary phase so that the overall effect of metal ions on the chromatographic behavior of merbarone may be complicated.

Qualitative evidence for complex formation was derived from perturbations in the UV absorption spectrum of merbarone in pH 7 phosphate buffer induced by the presence of Mg(II). However, the degree of association was inadequate to permit quantitative characterization according to traditional spectrophotometric techniques, such as the molar ratio method (12) and the method of continuous variations (13). Furthermore, the very low solubility of merbarone in aqueous and mixed organic-aqueous media precluded the use of potentiometric methods for monitoring complexation (14, 15). DMSO is not a suitable cosolvent for studying complexation since it is known to complex with metal ions (16).

Internal Standard

A structural analog, 3'-F-merbarone, proved to be a suitable internal standard to monitor losses in the recovery of merbarone during the sample preparation procedure. Its solubility characteristics, adsorption properties and UV spectrum were similar to that of merbarone and the compound eluted sufficiently late so that there was no interference with the parent drug or its plasma metabolite. Addition of the internal standard prior and subsequent to protein precipitation afforded standard curves with similar slopes indicating that there was no loss of drug in the samples prepared from plasma. The internal standard may be readily prepared from the reaction of 3-F-phenyl isocyanate and 2-thiobarbituric acid, which are commercially available, catalyzed by triethylamine in anhydrous peroxide-free dioxane at 80°C (17, 18).

Modifications of the Previous Method

Two deficiencies were identified in the earlier analytical method. The first was that corrosion of stainless steel tubing and fittings occurred with continued use of the mobile phase. The corrosion was identified to result from the inclusion of chloride ion in the eluent and did not recur when magnesium chloride was replaced with magnesium sulfate. The second complication was that the analytical columns used in the plasma assays had provided a short period of service, typically 350 plasma sample injections in spite of the use of inline filters and a guard column. The need for DMSO in the solution

used for the deproteinization evidently resulted in incomplete precipitation which may have contributed to the short column life. Furthermore, it is likely that magnesium hydroxide and magnesium carbonate formation was deleterious to column life. These premises provided the basis for the modifications of the mobile phase which were examined. Extension of the length of useful service of the columns was achieved through the addition of acetic acid and sodium dodecyl sulfate to the mobile phase.

Specificity of the Analytical Method

Support for the specificity of the analytical method was derived from studies with a similar isocratic system, column and mobile phase but using a diode array detector to permit the acquisition of UV spectra during chromatography. The similarity of UV spectra determined on the upslope, apex and downslope of the peak corresponding to merbarone in chromatograms of the plasma samples demonstrated that the peak was spectrally homogeneous. Moreover, an authentic sample of merbarone exhibited an identical retention time and good superposition of UV spectra determined at the apex of its chromatographic peak with that of merbarone in the patient plasma, indicating that the peak monitored in the samples corresponded to the parent drug.

The metabolite peak eluting prior to the drug appeared comparatively larger in the three-dimensional chromatogram, demonstrating that chromatograms determined at 306 nm do not adequately convey the relative concentration of this component. This is a consequence of significant differences in the spectral characteristics of these compounds; the metabolite exhibits an absorption maximum at 278 nm with a shoulder near 250 nm, while the absorption maxima of merbarone are at 306 and 236 nm.

Conclusion

In summary, this HPLC assay for merbarone in plasma permits the quantitation of 0.050 μg/ml; it is specific, reproducible and requires only 50 μl of plasma. Increasing the volume of plasma for sensitivity enhancement was not explored, since the present method was adequate to characterize the plasma pharmacokinetics in patients at the initial dose level of the phase I trial (96 mg/m²/day by civ for 5 days). The analytical method proved satisfactory for the quantitation of merbarone in urine, however, it was not suitable for quantitation of the polar metabolites in urine, since endogenous urinary constituents interfered with their chromatographic peaks. Therefore, a separate method is required for quantitating merbarone and its metabolites in urine. The mobile phase employed in these studies is novel, since it utilizes magnesium ions for complexing

merbarone and its related compounds. UV spectral changes demonstrated the formation of a weak complex between merbarone and magnesium ion.

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