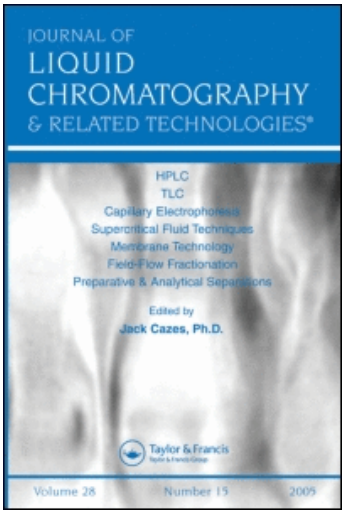

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Concurrent Determination of Merbarone and Its Urinary Metabolites by Reversed-Phase HPLC with Precolumn Phenolic Acetylation

Jeffrey G. Supko[a]; Louis Malspeis[a]
[a] Laboratory of Drug Discovery Research, Development National Cancer Institute Frederick Cancer Research Facility, Frederick, Maryland

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CONCURRENT DETERMINATION OF MERBARONE AND ITS URINARY METABOLITES BY REVERSED-PHASE HPLC WITH PRECOLUMN PHENOLIC ACETYLATION

JEFFREY G. SUPKO AND LOUIS MALSPEIS
Laboratory of Drug Discovery Research and Development
National Cancer Institute
Frederick Cancer Research Facility
Frederick, Maryland 21702-1201

ABSTRACT

Three prominent oxidative metabolites, 2-oxo-desthiomerbarone, 4'-hydroxymerbarone and 4'-hydroxy-2-oxo-desthiomerbarone are excreted in the urine of patients treated with merbarone (NSC 336628), an investigational anticancer agent. The highly polar phenolic metabolites were readily acylated using acetic anhydride in the presence of potassium carbonate without structurally altering the parent drug or 2-oxo-desthiomerbarone. Capacity factors of the derivatives were sufficiently enhanced to effect their separation from interfering endogenous urinary constituents under isocratic reversed-phase conditions. Chromatography was performed on a 4 μm Nova-Pak C_{18} column (3.9 mm x 15 cm) utilizing methanol-water (25:75, v/v) containing ammonium acetate (67 mM), acetic acid (33 mM), magnesium sulfate (40 mM) and sodium dodecyl sulfate (1 mM) as the mobile phase with UV detection at 293 nm. This method permitted the concurrent determination of merbarone and its three metabolites in a single urine sample, without preliminary isolation or purification procedures, by HPLC directly after an essentially quantitative, very rapid and phenol specific derivatization. Employing 100 μl of urine, calibration curves were constructed with analyte concentrations in the range 0.25-10.0 μg/ml, the lower limit being sufficient for quantitation in 24 hour pooled urines acquired during 7 days from cancer patients treated with the drug according to 5 day continuous intravenous infusion dosing regimens.

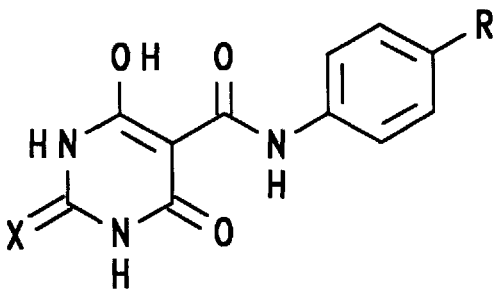
INTRODUCTION

Significant antitumor activity among barbituric acid derivatives has only been observed for those compounds which contain a 5-carboxanilide substituent (1-4). Although knowledge of their chemotherapeutic effects has existed since 1960 (3), recent interest developed after merbarone (MB; NSC 336628), 5-(N-phenylcarboxamido)-2-thiobarbituric acid, was found to exhibit exceptional cytotoxicity against several *in vivo* murine tumor models (2). Phase I clinical trials with cancer patients were initiated following a satisfactory preclinical evaluation (5).

Studies on the metabolism of MB in cancer patients treated according to 5 day continuous intravenous infusion (civ) schedules were conducted in our laboratories (6, 7). Relative to the drug, appreciable levels of three metabolites were evident in patient urines. These compounds were identified as 4'-hydroxy-2-oxo-desthio-merbarone (4'-OH-2-oxo-MB), 4'-hydroxymerbarone (4'-OH-MB) and 2-oxo-desthio-merbarone (2-oxo-MB), the chemical structures of which are shown in Figure 1. The presence of conjugative metabolites in urine or plasma samples could not be established, suggesting that MB biotransformation in humans is restricted to oxidative pathways. Plasma levels of 2-oxo-MB were much lower than the parent drug at all times during and after treatment and accumulation of the hydroxylated metabolites was inconsequential (8).

Metabolite quantitation in patient urines was of interest for evaluating the contribution of metabolite excretion toward overall drug elimination. In a previous paper (8), we reported an isocratic reversed-phase HPLC assay for MB in plasma. This method was employed for preclinical and clinical pharmacokinetic studies and initially for determining urine levels of the drug. Although 2-oxo-MB could have been concurrently assayed in either plasma or urine without significant modification of the method, the polar hydroxylated metabolites were not sufficiently resolved from early eluting endogenous urinary constituents to permit quantitation (7).

In this work, an HPLC method is described for the simultaneous determination of MB and its primary metabolites utilizing a facile precolumn derivatization procedure based upon the rapid, quantitative acetylation of phenolic compounds in aqueous alkaline solution with acetic anhydride (9). The hydroxylated metabolites were selectively acetylated, without affecting either the drug or 2-oxo-MB, forming species with enhanced retention times (R_T) under reversed-phase chromatographic conditions. Furthermore, the reaction was performed directly in urine, as preliminary isolation of the analytes and cleanup procedures after derivatization were not necessary.



COMPOUND	X	R
Merbarone	S	H
2-oxo-MB	O	H
4'-OH-MB	S	OH
4'-OH-2-oxo-MB	O	OH
4'-OAc-MB	S	$OCOCH_3$
4'-OAc-2-oxo-MB	O	$OCOCH_3$

FIGURE 1. Chemical structure of merbarone and derivatives.

MATERIALS AND METHODS

Reagents and Chemicals

Synthetic samples of merbarone (NSC 336628), 2-oxo-desthiomerbarone (NSC 366236), 4'-hydroxymerbarone (NSC 380960), 4'-hydroxy-2-oxo-desthiomerbarone, 4'-acetoxymerbarone and 4'-acetoxy-2-oxo-desthiomerbarone, prepared as previously described (7), were used as the analytical reference standards in these studies. The internal standard, 3'-F-merbarone (NSC 372106), was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Ammonium acetate, acetic acid and anhydrous potassium carbonate were analyzed reagents (J.T. Baker Chemical Co., Phillipsburg, NJ). Dodecyl sodium sulfate was purchased from Eastman Kodak (Rochester, NY). Analytical reagent magnesium sulfate heptahydrate (Mallinkrodt, Paris, KY) and certified A.C.S. acetic anhydride (Fisher Scientific, Fairlawn, NJ) were used. Glass distilled OmniSolv grade methanol (E.M. Science, Cherry Hill, NJ) and HPLC grade dimethylsulfoxide (DMSO) from Burdick and

Jackson Laboratories (Muskegon, MI) were used. Deionized, double distilled water was filtered through a 0.2 μm Nylon-66 filter (Rainin Instrument Co., Woburn, MA) before use.

Apparatus

Chromatography was performed using a model 114M pump (Beckman Instruments, Berkeley, CA), a WISP 712 automatic injector (Waters Associates, Milford, MA) and a variable wavelength Spectroflow 783 programmable absorbance detector (ABI Analytical, Kratos Division, Ramsey, NJ) containing a 12 μl flow cell (path length 8 mm) was used to monitor UV absorption. 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) and a 0.5 μm postinjector filter (Rainin Instrument). UV spectra during chromatography were obtained 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 500 μl sample loop.

Milligram quantities of the samples used for preparing stock solutions were weighed on a Cahn 25 electrobalance (Cahn Instruments, Cerritos, CA). Stock solutions were prepared in class A borosilicate glass volumetric flasks deactivated with 3% (v/v) Surfasil in toluene (Pierce Chemical Co., Rockford, IL). 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, Philadelphia, PA) at 12,000 x g.

Internal Standard Solutions

A 0.1 mg/ml stock solution of the internal standard, 3'-F-MB, was prepared in DMSO. An 800 μl aliquot of this solution was diluted to 5 ml with DMSO, providing the solution (16 μg/ml) used in the assay.

Urine Standards

A single stock solution containing MB, 2-oxo-MB, 4'-OH-MB, and 4'-OH-2-oxo-MB in DMSO was prepared such that the concentration of each compound was approximately 1.0 mg/ml. Pipetting drug-free urine (0.5-1.0 ml) into polypropylene microcentrifuge tubes and adding varying volumes of the analyte stock solution afforded standards containing 4.0, 6.0, 8.0 and 10.0 μg/ml with respect to the drug and each metabolite.

Serial dilution of the 4.0 μg/ml standard with drug-free urine afforded four additional solutions ranging in concentration from 0.25 to 2.0 μg/ml. These solutions were thoroughly mixed by vortexing for 1 min.

Sample Preparation

To 100 μl of urine in a polypropylene microcentrifuge tube, 25 μl of the internal standard solution (16 μg/ml) and 1.2 M aqueous potassium carbonate (25 μl) were pipetted. Immediately upon the addition of neat acetic anhydride (10 μl), the tube was capped and mixed on a vortex action stirrer for 1.0 min. After permitting it to stand for an additional 5 min, the sample was diluted with 400 μl of methanol-water (35:65, v/v) containing ammonium acetate (67 mM), acetic acid (33 mM), $MgSO_4 \cdot 7H_2O$ (40 mM), and sodium dodecyl sulfate (1 mM). The tube was again mixed by vortexing, centrifuged for 5 min and then transferred to a 250 μl polypropylene insert which was placed in the automatic sampler. An injection volume of 30 μl was employed. For urine samples containing analyte concentrations which exceeded the range of the standard curve, an aliquot of the sample was diluted with drug-free urine to make the total volume 100 μl.

Chromatographic Conditions

Isocratic reversed-phase chromatography was performed at ambient temperature with a methanol-water (25:75, v/v) mobile phase containing ammonium acetate (67 mM), acetic acid (33 mM), magnesium sulfate (40 mM), and sodium dodecyl sulfate (1 mM). The solution was degassed in an ultrasonic bath for 15 min prior to use. With a 1.0 ml/min flow rate, absorbance of the column effluent was monitored at a wavelength of 293 nm (5 nm bandwidth). The integrator was configured to report peak areas employing a 0.3 min peak width and a threshold setting of 2. Chromatograms were recorded using a 0.2 cm/min chart speed at an attenuation of 2.

Acceptable resolution was not achieved when a precolumn was incorporated in the system. For this reason, the following procedure was adopted for routinely regenerating the analytical column after approximately 100 samples were assayed. The column was reversed and flushed at 1.0 ml/min with water (300-500 ml) followed by methanol (300-500 ml), and then methanol-water (25:75, v/v) for one hour. Equilibrium with the analytical mobile phase was reestablished overnight.

Quantitation

On a daily basis, standard curves were constructed by plotting the peak area ratios of each analyte to the internal standard against the analyte concentration. The best fit straight line was determined by least squares regression using a weighting factor of inverse peak area ratio squared to calculate the slope, *y*-intercept and correlation coefficient. Analyte concentrations in unknown samples were calculated using results of the corresponding regression analysis.

Relative Recovery

The relative recovery of each analyte was determined from its calculated concentrations in the urine standards assayed during a 3.5 month period.

Optimization of Reaction Conditions

Aliquots of the 8.0 μg/ml urine standard were prepared and assayed as described above with the following exceptions: (1) the effect of potassium carbonate concentration was evaluated by employing solutions of the reagent which ranged from 0-3.0 M; (2) for each alkali concentration, the volume of acetic anhydride was varied from 0-25 μl. Samples for each set of reaction conditions were prepared in duplicate. The analyte to internal standard peak area ratios were calculated for the four components. If the individual peak area ratios deviated from the mean of the duplicate runs by more than 10%, a third trial was performed.

Stability of the Acetate Derivatives

Stability of the acetate derivatives of 4'-OH-MB and 4'-OH-2-oxo-MB in the assay solution was examined at ambient temperature for a period of 24 hours. Samples of the 8.0 μg/ml urine standard were individually prepared in duplicate as described above. The solutions were allowed to stand in the automatic sampler until injected at intervals of 0.5, 1.0, 2.0, 4.0, 8.0 and 24.0 hr subsequent to diluent addition.

RESULTS

Optimization of Reaction Conditions

The derivatization procedure was optimized by varying the amounts of potassium carbonate and acetic anhydride added to the urine standard containing 8.0 μg/ml of each analyte in the presence of internal standard. Reactions were initiated by adding

acetic anhydride to the alkalinized sample and immediately mixing for 1 min vigorously. Since preliminary experiments indicated that the reaction was extremely rapid at ambient temperature, effectively terminating within this time period, it was not necessary to alter the temperature and duration of mixing.

For the 20 variations in reaction conditions examined, there was no effect on the peak area ratios of MB and 2-oxo-MB relative to the internal standard, which were (mean ± SD) 2.612 ± 0.125 and 2.551 ± 0.107, respectively. The effect of potassium carbonate concentration and volume of added acetic anhydride on the acetylation of 4'-OH-MB and 4'-OH-2-oxo-MB in urine is illustrated in Figure 2. Maximum peak area ratios for the derivatives, 4'-acetoxymerbarone (4'-OAc-MB) and 4'-acetoxy-2-oxo-desthiomerbarone (4'-OAc-2-oxo-MB), relative to the internal standard occurred when the concentration of added alkalinizing reagent was 1.2 M with 5-10 μl of acetic anhydride. Magnesium hydroxide precipitation resulted upon dilution with mobile phase when 3.0 M potassium carbonate was used or 1.5 M alkali with less than 25 μl of acetic anhydride.

Except for the control run in which neither alkali or acetic anhydride were added, the pH of these solutions prior to anhydride addition was at least 10. The pH of reaction mixtures ranged from 4-6 approximately 5 min after the addition of acetic anhydride for runs in which the concentration of added potassium carbonate was 0.06-1.2 M. A significant diminishment in pH was not evident when alkali concentrations greater than 1.2 M were employed.

These observations were consistent with the two competing reactions that the anhydride is subject to in this system. Base catalyzed attack by the phenolic compound affords the acetate ester and an equivalent of acetic acid. However, the alkaline conditions required to effect this reaction also induces appreciable hydrolysis of the anhydride. Neutralization of the acetic acid formed in both reactions consumes hydroxide ions and the pH of the solution decreases. The desired reaction will be inhibited if the rate of pH reduction greatly exceeds that of esterification, resulting in less than quantitative derivatization of the phenolic compounds.

Urine Sample Preparation

Precolumn derivatization of the hydroxylated MB metabolites was performed directly in urine alkalinized with potassium carbonate at ambient temperature using acetic anhydride. The reaction was rapid, quantitative and specific in that MB, 2-oxo-MB and the internal standard (3'-F-MB) were not affected by conditions necessary for phenolic acetylation. After diluting the derivatized urine with a solution similar to the

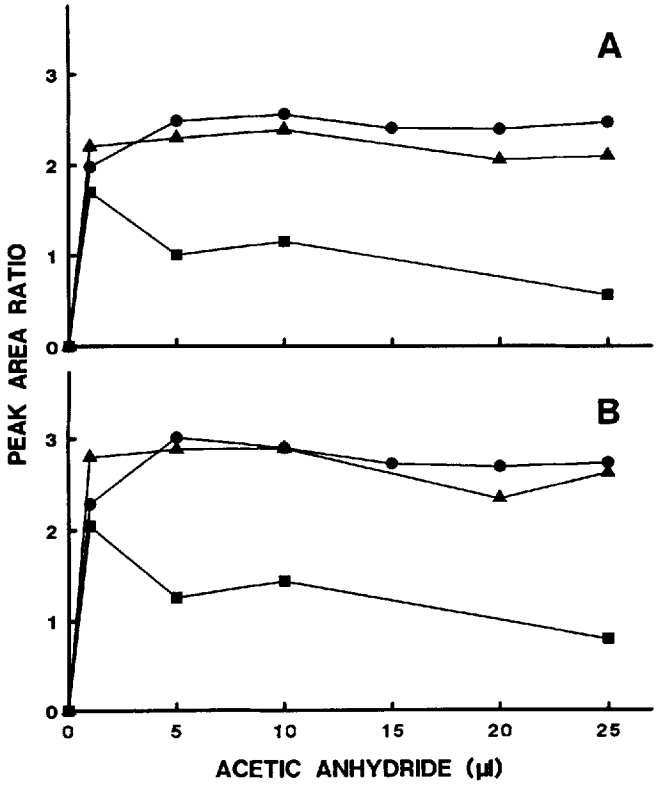


FIGURE 2. Optimization of the reaction conditions for acetylating (A) 4'-OH-MB and (B) 4'-OH-2-oxo-MB in urine. Derivative to internal standard chromatographic peak area ratios were determined after varying the potassium carbonate concentration and volume of acetic anhydride. Concentration of added potassium carbonate: 0.06 M (■), 0.60 M (▲) and 1.20 M (●).

mobile phase, but containing a higher fraction of methanol to adjust the solvent strength, the sample was chromatographed using a reversed-phase system with detection of the analytes by UV absorption at 293 nm. The chromatographic run time for a single sample was 20 min. Thus, approximately 10 hr was required to assay a standard curve, consisting of 8 urine standards plus an analyte free sample, and a typical series of patient urine specimens in duplicate. Derivative stability in the sample solution was sufficient to permit overnight assaying with an automatic injector.

Liquid Chromatography with Precolumn Acetylation

A chromatogram at 293 nm, determined with the diode array detection system, of directly injected patient urine acquired during the administration of MB by 24 hr civ at 1000 mg/m^2/day illustrates that peaks due to the drug (R_T 8.9 min) and 2-oxo-MB (R_T 7.6 min) are sufficiently resolved to permit their quantitation (Figure 3A). As indicated by comparison with a similarly acquired chromatogram of an aqueous solution containing the synthetically prepared compounds (Figure 3B), UV absorbing urinary constituents interfered with detection of the polar metabolites 4'-OH-2-oxo-MB and 4'-OH-MB, eluting near 1.4 and 1.8 min, respectively. The presence of the former metabolite was completely masked by the endogenous compounds. However, as shown in Figure 3C, derivatizing the alkalinized aqueous sample with acetic anhydride prior to chromatography shifted peaks associated with the phenolic metabolites to a region apparently free from interfering components. The complete absence of peaks at retention times corresponding to 4'-OH-2-oxo-MB and 4'-OH-MB demonstrates that the optimized reaction conditions achieved essentially quantitative derivatization.

Figure 4A shows a chromatogram of pretreatment urine assayed according to the derivatization procedure without internal standard using the analytical HPLC system with detection at 293 nm. The samples generally afforded clean chromatograms with no endogenous peaks which interfered with the drug, its metabolites or the internal standard. Chromatograms of urine standards containing each of the four analytes at concentrations of 0.25, 2.0 and 10.0 μg/ml are shown in Figures 4B-D. Pooled urine specimens, collected from a patient on the last day of a 190 mg/m^2 2 hr civ daily x 5 MB dosing regimen, chromatographed directly and after derivatization are shown in Figure 5. In general, the urine concentrations of 4'-OH-MB and 2-oxo-MB during therapy exceeded the upper range of the standard curves and were considerably greater than either MB or 4'-OH-2-oxo-MB. Therefore, some urine samples had to be assayed with and without dilution for quantitating each of the four analytes. Typical retention times (mean ± SD, n = 10) acquired from a series of samples assayed on a single day were: 4'-OAc-2-oxo-MB, 4.89 ± 0.20; 4'-OAc-MB, 6.63 ± 0.29; 2-oxo-MB, 8.33 ± 0.26; MB, 9.75 ± 0.23; and internal standard, 14.96 ± 0.37 min.

Specificity of the Analytical Method

The respective capacity factors of 4'-OH-2-oxo-MB and 4'-OH-MB in urine after derivatization with acetic anhydride were identical to synthetic samples of 4'-OAc-2-oxo-MB and 4'-OAc-MB (Figure 6). Chromatograms demonstrating assay specificity

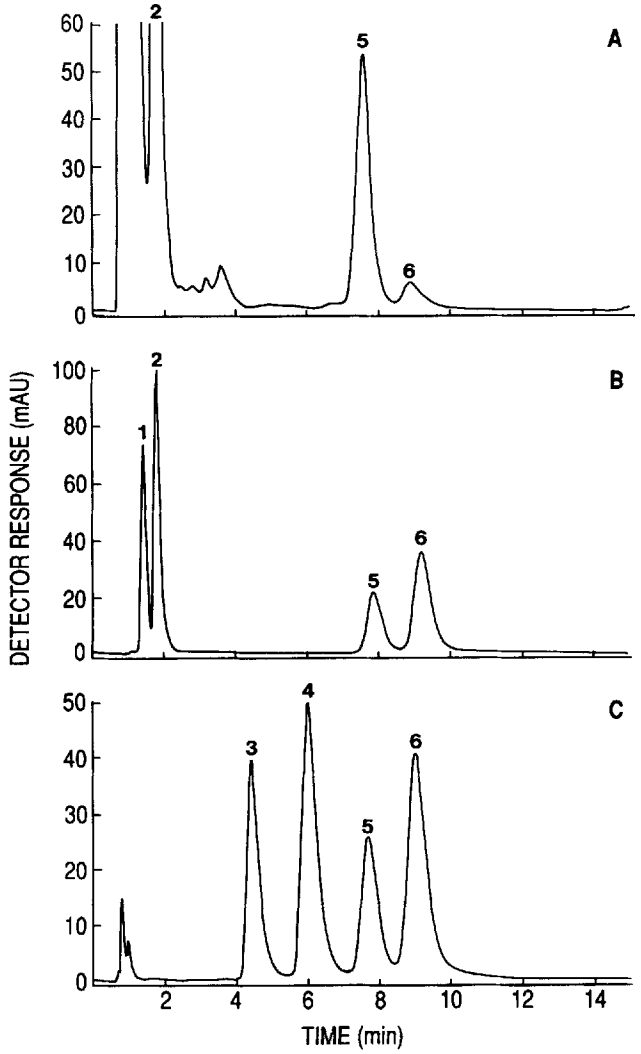


FIGURE 3. Liquid chromatograms determined at 293 nm with a diode array detector demonstrating the increased retention of the polar phenolic metabolites following precolumn acetylation. (A) Direct injection of 24 hr pooled patient urine collected on day 5 during the civ of MB at 1000 mg/m^2/day x 5. An aqueous solution containing synthetically prepared samples of merbarone and its primary urinary metabolites chromatographed before (B) and after (C) acetylation with acetic anhydride in the presence of potassium carbonate. Chromatographic peaks: **1**, 4'-OH-2-oxo-MB (1.4 min); **2**, 4'-OH-MB (1.8 min); **3**, 4'-OAc-2-oxo-MB (4.5 min); **4**, 4'-OAc-MB (6.0 min); **5**, 2-oxo-MB (7.7 min); **6**, merbarone (9.2 min).

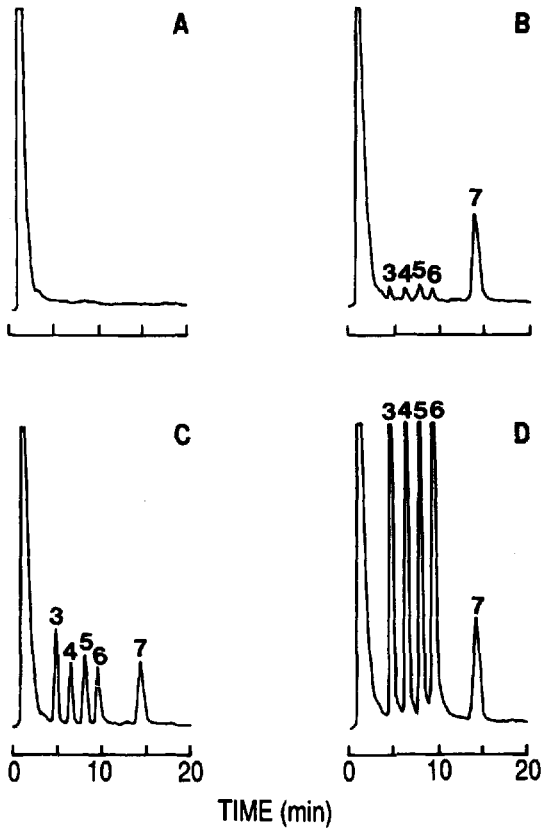


FIGURE 4. Representative liquid chromatograms of urine standard curve samples. (A) Analyte and internal standard free urine; (B) analytes, 0.25 μg/ml; (C) analytes, 2.0 μg/ml; (D) analytes, 10.0 μg/ml. Chromatographic peaks: **3**, 4'-OAc-2-oxo-MB; **4**, 4'-OAc-MB; **5**, 2-oxo-MB; **6**, merbarone; **7**, internal standard (3'-F-MB).

were determined with patient pooled urines obtained on day 5 during the 24 hr civ of 1000 mg/m^2/day doses of the drug. A three-dimensional chromatogram from 0-15 min of derivatized pretreatment urine exhibited a small peak at about 7 min, which did not exhibit significant absorption above 260 nm, and was otherwise free of interfering components in the region where the four analyte peaks and internal standard elute (Figure 7A). A similar chromatogram of pooled urine obtained on day 5 during dosing

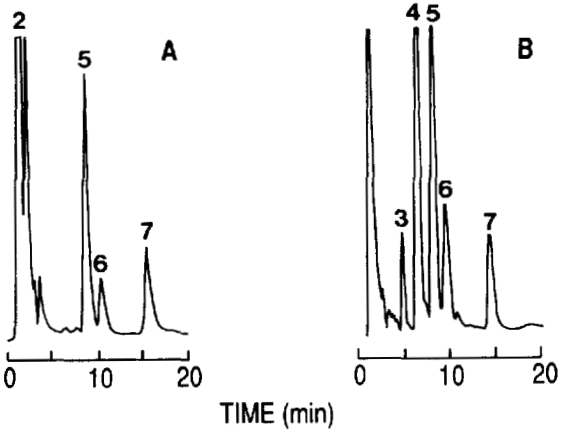


FIGURE 5. Chromatograms of patient urine obtained on day 5 during the 2 hr infusion of merbarone, 190 mg/m^2/day x 5. (A) Sample chromatographed directly and (B) following precolumn acetylation of the phenolic metabolites.

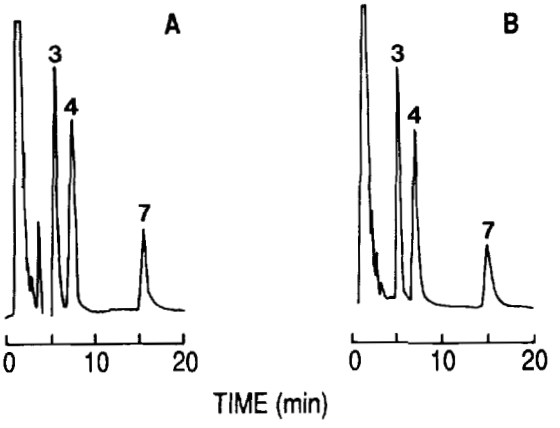


FIGURE 6. Chromatograms of drug-free urine spiked with synthetic samples of (A) 4'-OAc-2-oxo-MB and 4'-OAc-MB injected directly and (B) 4'-OH-2-oxo-MB and 4'-OH-MB assayed with precolumn derivatization. The concentration of each analyte was 6 μg/ml.

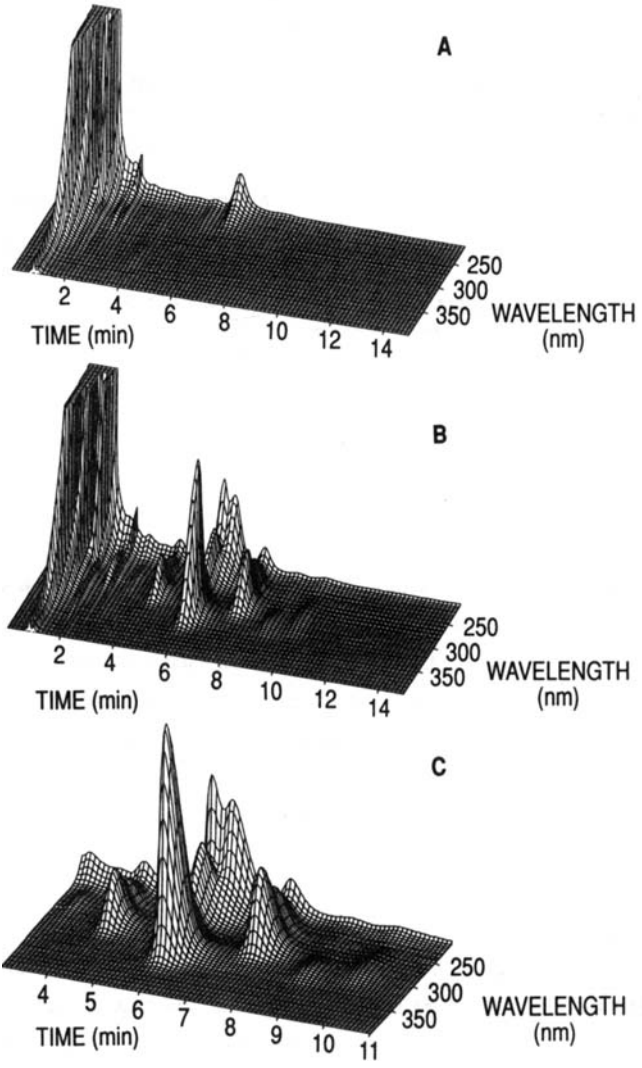


FIGURE 7. Three-dimensional chromatograms of (A) a pretreatment patient urine specimen, and (B) 24 hr pooled urine acquired on day 5 during the civ of merbarone, 1000 mg/m^2/day x 5, assayed according to the derivatization procedure. (C) View of the region from 3-11 min of the derivatized sample with enhanced sensitivity.

TABLE 1

Linear Regression Parameters from Standard Curves for Merbarone and its Major Metabolites in Human Urine[a]

Analyte	Slope (ml/μg)	y-intercept	r[b]
4'-OH-2-oxo-MB	0.3802 ± 0.0166	0.0487 ± 0.0296	0.9981 ± 0.0017
4'-OH-MB	0.3158 ± 0.0156	0.0022 ± 0.0253	0.9977 ± 0.0011
2-oxo-MB	0.3274 ± 0.0175	0.0274 ± 0.0203	0.9970 ± 0.0024
MB	0.3384 ± 0.0252	-0.0064 ± 0.0127	0.9984 ± 0.0008

[a] Mean ± standard deviation, number of replicates = 10.
[b] Correlation coefficient.

demonstrates the applicability of the acylation procedure to patient urine, affording chromatograms in which the esters, 2-oxo-MB and the parent drug are adequately resolved (Figure 7B). Figure 7C shows the region containing the peaks of interest with enhanced visualization. Parity between UV spectra of each metabolite derivative and the corresponding synthetic compound was ascertained from superpositions of spectra determined at the chromatographic peak apexes (data not presented). Similarly, the homogeneity of eluted components was demonstrated by comparing the normalized absorption spectra acquired on the upslope, apex and downslope of each chromatographic peak.

Calibration Curves

Standard curves for 4'-OH-2-oxo-MB, 4'-OH-MB, 2-oxo-MB and MB were constructed by plotting analyte to internal standard peak area ratios against urine concentration. Best fit lines of the calibration curves, encompassing concentrations from 0.25-10.0 μg/ml for each compound, were obtained by linear regression analysis employing a weighting factor of reciprocal peak area ratio squared. Mean values for the slopes, y-intercepts and correlation coefficients determined from 10 standard curves acquired on separate days are presented in Table 1.

TABLE 2

Relative Recovery and Reproducibility of the Analytical Method for Merbarone and Its Major Metabolites in Human Urine[a]

Amount Added (μg/ml)	Mean Amount Found (μg/ml)	Recovery (%)	Coefficient of Variation (%)
4'-Hydroxy-2-oxo-desthiomerbarone			
0.2520	0.2525	100.2	3.89
0.5030	0.4921	97.8	5.51
1.006	1.066	106.0	8.94
2.012	2.016	100.2	4.54
4.025	4.005	99.5	3.74
6.025	6.130	101.7	3.85
8.018	8.005	99.8	4.69
10.02	9.863	98.4	3.36
4'-Hydroxymerbarone			
0.2511	0.2538	101.1	3.62
0.5022	0.5027	100.1	8.80
1.004	1.017	101.2	7.81
2.009	1.947	96.9	5.18
4.018	3.971	98.8	2.83
6.014	6.239	103.7	2.65
8.003	8.203	102.5	5.10
10.00	10.03	100.3	2.31
2-Oxo-desthiomerbarone			
0.2508	0.2574	102.6	7.35
0.5016	0.4935	98.4	9.70
1.003	1.033	103.0	10.86
2.006	1.984	100.2	5.32
4.013	3.976	98.9	3.51
6.007	6.182	102.9	4.41
7.994	8.113	101.5	4.66
9.992	9.970	99.8	2.81
Merbarone			
0.252	0.2567	101.8	3.01
0.504	0.4954	98.3	6.24
1.008	1.014	100.5	4.71
2.017	1.963	97.3	4.04
4.034	3.991	98.9	3.10
6.038	6.270	103.8	3.51
8.035	8.237	102.5	4.34
10.04	10.04	99.9	2.86

[a] Number of replicates = 10.

Relative Recovery and Assay Reproducibility

Urine standards prepared on 10 days during a 3.5 month period from a single stock solution containing each of the metabolites and MB were chromatographed with a single analytical column. The relative recoveries of the four analytes from urine and reproducibility for the range of concentrations which defined the standard curves are summarized in Table 2. Coefficients of variation for the replicate assays ranged from 3.36-8.94% for 4'-OH-2-oxo-MB, 2.31-8.80% for 4'-OH-MB, 2.81-10.86% for 2-oxo-MB, and 2.86-6.24% for MB. Inasmuch as these calculated concentrations were obtained during an extended period the analytical method is considered to be reproducible. Relative recoveries of the amount of each compound added to urine ranged from 97.8-106.0% for 4'-OH-2-oxo-MB, 96.9-103.7% for 4'-OH-MB, 98.4-103.0% for 2-oxo-MB, and 98.3-103.8% for the drug. The coefficient of variation for the averaged slopes of the linear regression curves were as follows: 4'-OH-2-oxo-MB, 4.36%; 4'-OH-MB, 4.94%; 2-oxo-MB, 5.34%; and MB, 7.45%. Accordingly, the assay proved to be consistent during this period.

DISCUSSION

Preliminary information indicated that only 1-2% of the MB administered to humans was eliminated by renal excretion as unchanged drug. However, the presence of several prominent nonendogenous peaks preceding the drug in liquid chromatograms of patient urine specimens suggested that MB was subject to extensive biotransformation (7). Upon isolating these components from urine by solid phase extraction, it became evident that there were three major metabolites, subsequently characterized as 2-oxo-MB, 4'-OH-MB and 4'-OH-2-oxo-MB. The availability of synthetic samples of these compounds, prepared for corroborating spectroscopic structural assignments, facilitated the development of an analytical method for urinary metabolite quantitation.

As previously reported (8), plasma levels of MB were monitored by directly loading plasma deproteinized with methanol-DMSO (82:18, v/v) onto a 4 μm Nova-Pak C_{18} (15 cm x 3.9 mm) HPLC column. Separations were achieved using an isocratic mobile phase 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, with detection by UV absorption at 306 nm. Under these conditions, both MB and its desulfurated metabolite, 2-oxo-MB, were adequately separated from each other and endogenous constituents in

urine samples chromatographed directly. However, the more polar hydroxylated metabolites, 4'-OH-2-oxo-MB and 4'-OH-MB, were insufficiently retained and eluted in the presence of interfering urinary components near the solvent front. In fact, visualization of 4'-OH-2-oxo-MB became apparent only after exhaustive purification to remove urinary substituents as it was masked by endogenous compounds (7).

Studies were therefore undertaken to develop a method for simultaneously assaying MB and its three metabolites in urine. Initial efforts were directed toward modification of the existing HPLC conditions to separate the hydroxylated metabolites from early eluting interferences. However, changes in the composition of the mobile phase and/or employing two analytical columns in tandem were ineffective. Gradient elution was rendered inconvenient by the prolonged time required to reestablish equilibrium of the system between successive injections.

Considering the chromatographic properties of MB analogs synthesized during earlier investigations suggested the feasibility of precolumn derivatization for effecting capacity factor enhancement (7). Specifically, 4'-OAc-MB and 4'-OAc-2-oxo-MB, prepared as precursors for the phenolic metabolites, eluted in a chromatographic region free from interfering components with respective R_T of 4.5 and 6.2 min. Accordingly, we undertook the development of a liquid chromatographic assay employing prior conversion of 4'-OH-MB and 4'-OH-2-oxo-MB to the less polar phenylacetates.

The acetylation of phenols in alkaline aqueous solution, as described by Chattaway (9), presented a convenient method to derivatize the metabolites directly, without preliminary extraction from urine. In the reported procedure, phenolic compounds were dissolved with a 50% molar excess of aqueous sodium hydroxide, which was sufficient to leave the medium slightly alkaline upon completion of the reaction. A 25% molar excess of acetic anhydride was then added to the ice cold solution and the mixture immediately shaken. The reaction was apparently instantaneous and quantitative.

Preliminary experiments to assess the applicability of this procedure were conducted utilizing an aqueous solution of synthetic 4'-OH-MB. Potassium hydroxide, at a final concentration of 0.5 M, was employed as the alkalinizing agent to avoid the potential formation of an insoluble complex with sodium ions (10, 11). Liquid chromatography indicated that essentially quantitative acetylation was achieved, however, the derivative peak appeared badly tailed. An incomplete reaction was realized upon decreasing the alkali concentration 10-fold. For this reason, the effectiveness of other bases to catalyze the acetylation of 4'-OH-MB were examined.

Although distortion of the derivative peak did not occur when the reaction was performed in the presence of sodium bicarbonate, complete conversion of the substrate was not achieved. Potassium carbonate was found to be an acceptable reagent in all respects.

The applicability of this procedure for concurrently assaying the drug and its major urinary metabolites was initially demonstrated by similarly treating urine obtained from a patient during the infusion of MB. Comparison of chromatograms determined before and after derivatization suggested that the phenolic metabolites were converted to the corresponding esters without affecting the drug or 2-oxo-MB. Inasmuch as the analyte peaks were relatively well resolved from each other and urinary constituents, sample cleanup procedures were apparently not necessary. The need for removing excess derivatizing agent and resultant degradation products was avoided, since acetic anhydride is transparent at the wavelength used for detection.

The isocratic mobile phase developed for the plasma assay was used in the present method with minor modification of the methanol strength, which was systematically varied for optimal resolution. Estimation of the chromatographic parameters and visual inspection indicated that the best separation was achieved with a 25% methanolic eluent at a flow rate of 1.0 ml/min. Significant improvements in resolution were not observed as the flow rate was varied.

The wavelength used for detection was 293 nm, selected as being intermediate between the wavelength of maximum absorbance in the mobile phase for the 2-thio compounds (MB and 4'-OAc-MB) at 306-308 nm and the 2-oxo compounds (2-oxo-MB and 4'-OAc-2-oxo-MB) at 278-279 nm. The compound used as an internal standard, 3'-F-MB, was also employed in the plasma assay. Components interfering with the internal standard were not apparent in chromatograms of patient predose urine and specimens acquired during and after treatment with the drug contained no interfering metabolites.

Retention times of the drug, metabolites and internal standard generally did not vary greatly for a given column. Unlike the plasma assay, a precolumn C_{18} cartridge was not employed as it adversely affected peak resolution, thus, the only protection for the analytical column was an inline postinjector filter. A procedure was therefore adopted to flush and regenerate each analytical column on a weekly basis. Three columns were alternately used for the analysis of approximately 1500 urine samples without significant deterioration of peak resolution.

In summary, this HPLC assay permits the quantitation of MB and its three principal urinary metabolites in a single human urine specimen. The method utilizes precolumn derivatization of the hydroxylated metabolites in order to effect their separation from interfering endogenous constituents. Chromatography was carried out under reversed-phase isocratic conditions and the four compounds were monitored by UV detection at 293 nm. Employing 100 μl of urine, the lowest concentration on calibration curves for each of the analytes was 0.25 μg/ml. This level of sensitivity was adequate for quantitating these compounds in urine samples obtained from all of the patients participating in the phase I trial, and therefore, no effort was made for its enhancement. The method was shown to be specific and reproducible.

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REFERENCES

1. Driscoll, J. S., Nannette, R. M., Quinn, F. R., Davignon, J. P., Ing, R., Abbott, B. J., Congleton, G. and Dudeck, L., Psychotropic Drugs as Potential Antitumor Agents: a Selective Screening Study, Cancer Treat. Rep., 62, 45, 1978.

2. Brewer, A. D., Minatelli, J. A., Plowman, J., Paull, K. D. and Narayaman, V. L., 5-(N-Phenylcarboxamido)-2-thiobarbituric acid (NSC 336628), a Novel Potential Antitumor Agent, Biochem. Pharmacol., 34, 2047, 1985.

3. Ukita, T., Kato, Y., Hori, M. and Nishizawa, H., On the Anti-tumor Activity of Nitrogenous Cyclic β-Diketones, Chem. Pharm. Bull., 8, 1021, 1960.

4. Ukita, T. and Mizuno, D., In Vitro Screening of Tricarbonylmethane and Related Compounds for Their Anti-tumor Effect by Cylinder Agar Plate (CAP) Method, Chem. Pharm. Bull., 8, 1016, 1960.

5. Glover, A., Chun, H. G., Kleinman, L. M., Cooney, D. A., Plowman, J., Grieshaber, C. K., Malspeis, L. and Leyland-Jones B., Merbarone: An Antitumor Agent Entering Clinical Trials, Investigational New Drugs, 5, 137, 1987.

6. Supko, J. G., Grever, M. R., Balcerzak, S. P., Kraut, E. H., Staubus, A. E. and Malspeis, L., Spectral Characterization and Quantitation of the Principal Metabolites of Merbarone (NSC 336628) in the Urine of Patients, Proc. Am. Assoc. Cancer Res., 29, 189, 1988.

7. Supko, J. G. and Malspeis, L., Characterization of the Urinary Metabolites of Merbarone in Cancer Patients, Drug Metab. Dispos., 19, 263, 1991.

8. Malspeis, L. and Supko, J. G., Liquid Chromatographic Determination of Merbarone (NSC 336628) in Plasma with Mg(II) as a Mobile Phase Modifier, J. Liquid Chromatogr., 13, 1301, 1990.

9. Chattaway, F. D., Acetylation in Aqueous Alkaline Solution, J. Chem. Soc., 2495, 1931.

10. NCI Investigational Drugs - Pharmaceutical Data 1987, Pharmaceutical Resources Branch, National Cancer Institute, NIH Publication No. 88-2141, Revised November, 1987.

11. Merbarone NSC 336628, Clinical Brochure, National Cancer Institute, Division of Cancer Treatment, Bethesda, MD., October, 1985.