*Journal of Chromatography*, 415 (1987) 148–155
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3482

## Note

# Gas chromatographic analysis of metabolites of the cell differentiating agent hexamethylene bisacetamide

MERRILL J. EGORIN*, ELEANOR G. ZUHOWSKI and M.S. BALACHANDRAN NAYAR

*Division of Developmental Therapeutics, University of Maryland Cancer Center, 655 West Baltimore Street, Baltimore, MD 21201 (U.S.A.)*

and

PATRICK S. CALLERY

*Department of Medicinal Chemistry/Pharmacognosy, School of Pharmacy, University of Maryland, 655 West Baltimore Street, Baltimore, MD 21201 (U.S.A.)*

(First received June 24th, 1986; revised manuscript received October 29th, 1986)

Hexamethylene bisacetamide (HMBA, NSC-95580) is a compound known to induce in vitro morphological and functional differentiation of murine and human leukemic and solid tumor cell lines [1–13]. Among the class of agents that have the potential for inducing the differentiation of tumor cells and which represent an exciting and novel approach to the chemotherapy of neoplasia [14–19] is HMBA which has a number of characteristics which render it of greatest potential clinical use. HMBA was selected for introduction into clinical trials because, of a series of bisacetamides tested, it approached maximum differentiation potency [1–5]. In addition, HMBA differs from differentiating agents such as dimethyl sulfoxide and N-methylformamide that have undergone previous clinical evaluation [20–27] in that carefully conducted clinical and pharmacokinetic studies have documented the ability to achieve concentrations of HMBA in patient plasma equal to the concentrations required for induction of differentiation in vitro [28,29]. On the other hand, administration of HMBA to humans has not proven devoid of adverse effects [28,30]. At HMBA dosages greater than or equal to 33.6 $g/m^2$ per day metabolic acidosis and neurotoxicity occur as dose-limiting toxicities. Platelet count suppression, although not dose-limiting, also occurs with HMBA therapy.

In view of the fact that the metabolism of HMBA was undefined, and with the

0378-4347/87/$03.50 

belief that elucidation of such metabolism would enhance studies of mechanism of action and etiology of toxicities we undertook the identification of HMBA metabolites found in humans. These studies, which utilized electron ionization and chemical ionization gas chromatography–mass spectrometry (GC–MS) led to the identification of five metabolites of HMBA, including the major metabolite 6-acetamidohexanoic acid, the monodeacetylated product N-acetyl-1,6-diaminohexane, the bis-deacetylated diamine 1,6-diaminohexane, and the amino acid 6-aminohexanoic acid and its lactam caprolactam [31]. Recognizing the potential importance of quantifying the amounts of each of these metabolites in body fluids of patients treated with HMBA or in cells and tissue culture media from in vitro studies led us to develop a GC analysis which would allow the routine and sensitive assay of HMBA metabolites in biological samples.

## EXPERIMENTAL

### *Materials*

1,6-Diaminohexane, 6-acetamidohexanoic acid, 6-aminohexanoic acid, acetic anhydride, trifluoroacetic anhydride, 2,2,2-trifluoroethanol, cadaverine and 1,2-diphenylethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). N-Acetyl-1,6-diaminohexane hydrochloride was synthesized with a modification of the method described by Callery et al. [31] and Tabor et al. [32].

### *Procedure*

Triplicate 100-$\mu$l samples of plasma or urine, containing known concentrations of N-acetyl-1,6-diaminohexane and 6-acetamidohexanoic acid, were mixed with 50 $\mu$l of 6 m*M* 1,2-diphenylethylamine internal standard. Similarly, triplicate 100-$\mu$l samples, containing known concentrations of 1,6-diaminohexane and 6-aminohexanoic acid, were mixed with 50 $\mu$l of 3 m*M* cadaverine internal standard. Ethanol (900 $\mu$l) was added to the mixtures to denature proteins. After vortexing and centrifuging for 10 min at 10 000 *g*, 700 $\mu$l of the resulting supernatant were transferred to a 1-ml derivatization vial and were evaporated to dryness under nitrogen. Trifluoroacetic anhydride (200 $\mu$l) was added to the dried residue and the mixture was heated at 80°C for 5 min. After cooling, 100 $\mu$l of 2,2,2-trifluoroethanol were added to the derivatization vial and the mixture was heated at 80°C for an additional 30 min. This final reaction mixture was cooled and evaporated to dryness under nitrogen. The residue was redissolved in 800 $\mu$l of ethyl acetate and 1 $\mu$l of the resulting solution was injected into a Hewlett-Packard 5840A gas chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.), fitted with a 1.8 m×2 mm I.D. glass column containing 3% SP-2250-DB on 100–120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). For analysis of N-acetyl-1,6-diaminohexane and 6-acetamidohexanoic acid, the oven was maintained at 180°C. For analysis of 1,6-diaminohexane and 6-aminohexanoic acid, the oven was maintained at 140°C. In all analyses, the injection port was maintained at 255°C and nitrogen, at a flow-rate of 30 ml/min, was used as a carrier gas. Detection was accomplished with a nitrogen–phosphorus detector that was maintained at 275°C with an air flow-rate of 90 ml/min, a hydrogen flow-rate of 3.5 ml/min and

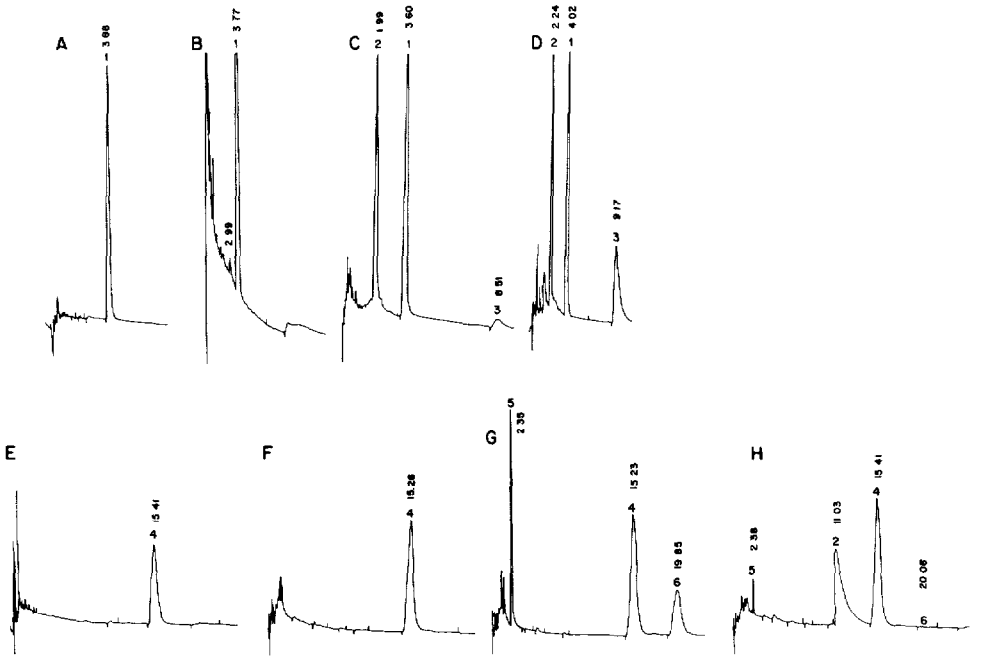


Fig. 1. Chromatograms of control plasma (A, E) and urine (B, F) to which were added 1,2-diphenylethylamine (1), cadaverine (4), 1,6-diaminohexane (5) or 6-aminohexanoic acid (6) and of plasma (C, G) and urine (D, H) from a patient after administration of hexamethylene bisacetamide. Peaks 2 and 3 represent 6-acetamidohexanoic acid and N-acetyl-1,6-diaminohexane, respectively.

a bead voltage of 16–18 V. Peaks were recorded and integrated with a Hewlett-Packard 5840A GC terminal. Concentrations of each metabolite were calculated by comparison of the area of the metabolite peak with that of the internal standard peak in each sample.

## RESULTS

With the sample processing and chromatographic conditions described herein, N-acetyl-1,6-diaminohexane and 6-acetamidohexanoic acid were well resolved from each other as well as from the 1,2-diphenylethylamine internal standard (Fig. 1). The retention times of 6-acetamidohexanoic acid, 1,2-diphenylethylamine and N-acetyl-1,6-diaminohexane were 1.99, 3.82 and 8.84 min, respectively. Similarly, 1,6-diaminohexane and 6-aminohexanoic acid were well resolved from each other as well as from the cadaverine internal standard (Fig. 1). The retention times of 1,6-diaminohexane, cadaverine and 6-aminohexanoic acid were 2.35, 15.30 and 19.95 min, respectively. There were no endogenous plasma or urinary materials which interfered with the determination of any of these compounds (Fig. 1). With the method as described, the limit of detection was 0.0625 m*M* for each metabolite studied. When peak-area ratios were used to evaluate detector response and to generate standard curves, linear relationships were observed over the concentration ranges 0.125–6.25 m*M* (Fig. 2). For plasma or

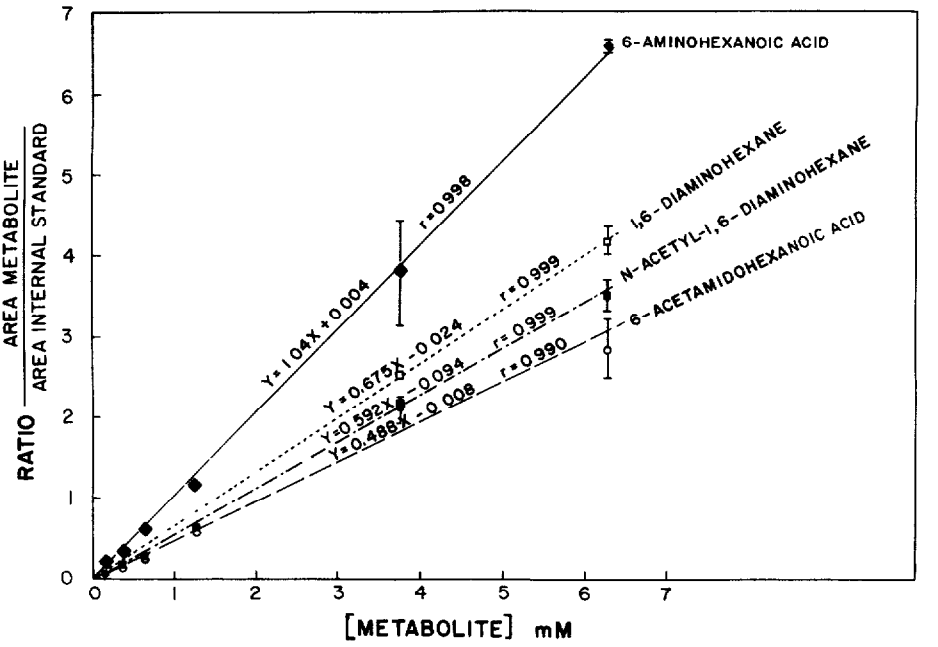


Fig. 2. Standard curves of individual metabolites of hexamethylene bisacetamide. Points represent means of triplicate samples. Error bars display S.D. Error bars for concentrations $< 3.75$ m$M$ have been omitted for clarity.

urine containing 0.625 m$M$ metabolite concentrations the coefficients of variation for N-acetyl-1,6-diaminohexane, 6-acetamidohexanoic acid, 1,6-diaminohexane and 6-aminohexanoic acid were 12.4, 5.1, 5.8 and 7.0%, respectively. Similar values were obtained when triplicate samples containing 3.75 m$M$ metabolite were analyzed. When standard curves were analyzed by linear regression, correlation coefficients were greater than or equal to 0.990 for each metabolite studied (Fig. 2).

There was no apparent effect of the sample matrix from which the metabolites were analyzed since simultaneously analyzed standard curves from distilled water, plasma and urine were indistinguishable. When the same 0.625 m$M$ or 3.75 m$M$ sample was analyzed on successive days, the day-to-day variation was 9.8, 5.2, 4.1 and 2.1% for N-acetyl-1,6-diaminohexane, 6-acetamidohexanoic acid, 1,6-diaminohexane and 6-aminohexanoic acid, respectively.

To validate the applicability of this GC method to biological samples, it was used to analyze plasma and urine samples from an 83-year-old patient with prostatic carcinoma who had received a five-day continuous intravenous infusion of HMBA at a dosage of 43.2 g/m$^2$ of body surface area per day. Heparinized, venous blood samples were collected before the HMBA infusion and at multiple times during and after the end of the infusion. Plasma, obtained by centrifuging the blood at 1000 $g$ for 10 min, was immediately frozen and stored at $-20$°C until analyzed. Urine was collected as voided, stored at 4°C and pooled as 4-h collections for the first 24 h. At the end of each collection, the volume of urine was

measured immediately and a portion of the urine was frozen and stored at −20°C until analyzed. Concentrations of HMBA in these samples were analyzed by GC as described previously [29,30].

Plasma concentrations of N-acetyl-1,6-diaminohexane and 6-acetamidohexanoic acid increased during the first 12–24 h of the HMBA infusion and thereafter remained relatively constant (Fig. 3). At most times, the concentration of 6-acetamidohexanoic acid exceeded that of N-acetyl-1,6-diaminohexane by two- to three-fold. At no time during the HMBA infusion did concentrations of either metabolite exceed those of parent compound, however, at steady-state, concentrations of 6-acetamidohexanoic acid were consistently in the range 0.5–0.9 m*M*. At no time during or after the HMBA infusion were plasma concentrations of 1,6-diaminohexane or 6-aminohexanoic acid greater than the limit of detection of our assay method. After completion of the HMBA infusion, concentrations of HMBA declined monoexponentially with a half-life of 5.65 h (Fig. 3). In contrast, concentrations of N-acetyl-1,6-diaminohexane and 6-acetamidohexanoic acid did not decline during the first 4–6 h after infusion.

Urinary excretion of N-acetyl-1,6-diaminohexane and 6-acetamidohexanoic acid was substantial, although in each case it was less than that of parent compound (Fig. 4). During the first 24 h of drug infusion, urinary excretion of these two compounds accounted for 7.7–12.6% of the daily dose of HMBA and when considered together accounted for only slightly less than the 28.4% excretion of dose represented by parent compound.

## DISCUSSION

HMBA has been introduced into phase I clinical trials with the hope of developing an antineoplastic agent with a mechanism of action that differs from the traditional cytotoxic effects exploited in the use of standard and most investigational antitumor drugs [14–19,34]. Although in vitro studies have documented numerous instances of the ability of HMBA to induce differentiation of tumor cell lines [1–13], there is much less evidence of in vivo activity [34]. In addition, phase I trials have carefully documented metabolic acidosis and neurotoxicity as dose-limiting toxicities associated with HMBA dosages $\geq$ 33.6 g/m$^2$ per day [28,30]. Our previous demonstration of multiple acidic and amine metabolites of HMBA [31] should allow a more rational investigation of both the discrepancy between in vitro and in vivo activity of HMBA and the toxicities attendant upon HMBA use. The GC method presented in this paper allows a quantitative rather than a qualitative approach to these issues.

The most obvious application of the method will be to define the plasma and urinary concentrations of HMBA metabolites in patients treated with various doses of HMBA. From the data in the single patient presented in this paper, it appears that 6-acetamidohexanoic acid is the major metabolite of HMBA present in plasma. This extends our earlier observation that this compound represents the major urinary metabolite of HMBA [31]. However, quantification of plasma concentrations of 6-acetamidohexanoic acid implies that this acid alone cannot explain the entire anion gap associated with HMBA use, since concentrations of

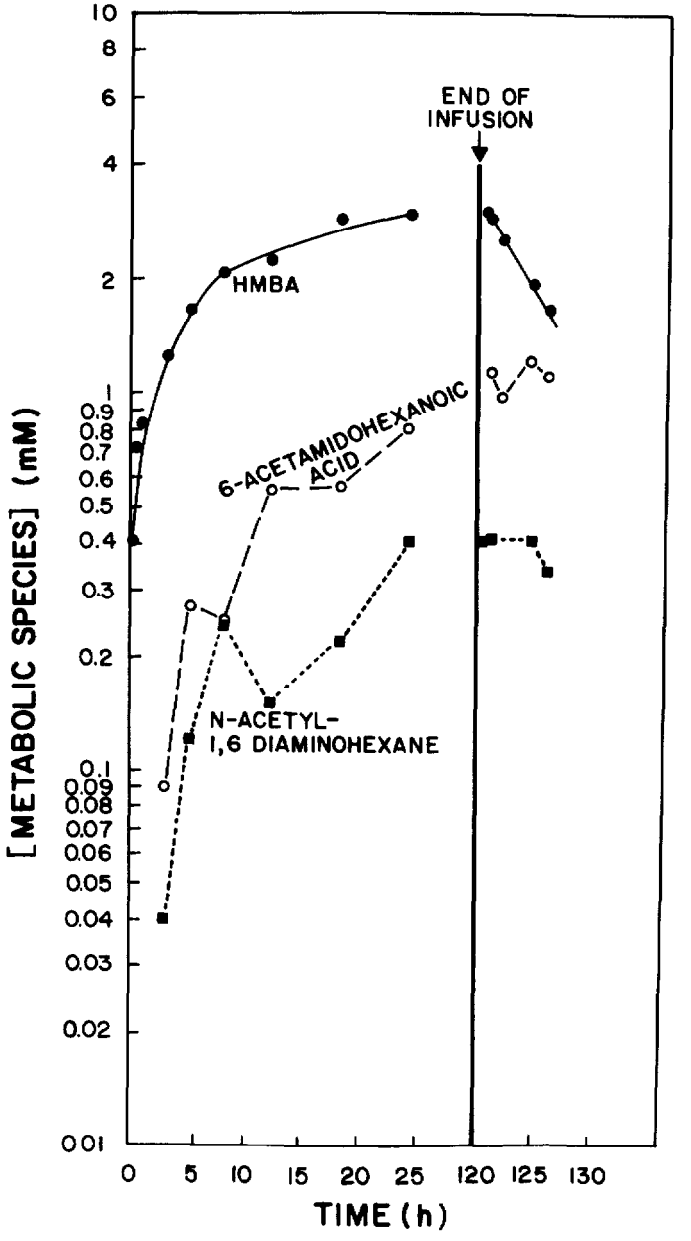


Fig. 3. Plasma concentrations of hexamethylene bisacetamide (HMBA), 6-acetamidohexanoic acid and N-acetyl-1,6-diaminohexane in the plasma of an 83-year-old patient with prostatic cancer who was treated with hexamethylene bisacetamide as a five-day continuous intravenous infusion at a dosage of 43.2 g/m$^2$ per day.

6-acetamidohexanoic acid only approached 1 m*M*, i.e. is far less than the 6–8 mequiv./l anion gap associated with HMBA toxicity. Current studies in our laboratory are extending these observations by measuring the concentrations of 6-acetamidohexanoic acid in the plasma of each patient treated in our phase I trial

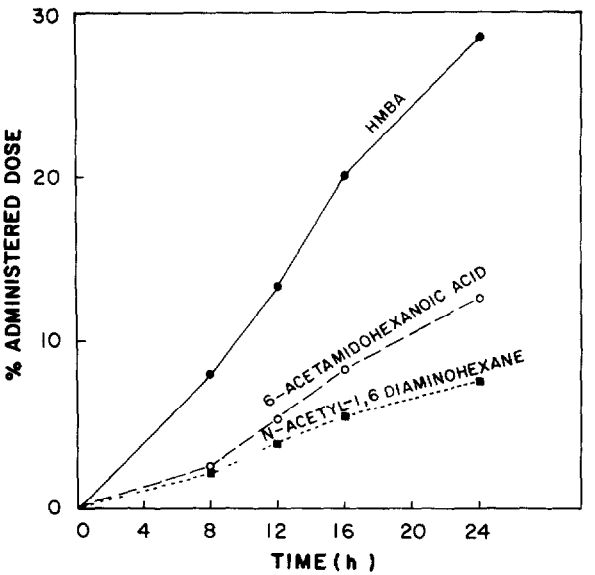


Fig. 4. Urinary excretion of hexamethylene bisacetamide (HMBA), 6-acetamidohexanoic acid and N-acetyl-1,6-diaminohexane by the patient described in the legend to Fig. 3.

[29,30]. This will determine if the patient presented in this paper is representative and should allow definition of any relationships between HMBA dose and plasma concentrations of 6-acetamidohexanoic acid.

The facility of the method described for measurement of 6-acetamidohexanoic acid and the fact that it employs the same GC column as does the analysis of HMBA [29,30] makes the method feasible to monitor plasma concentrations of both parent compound and metabolites. This ability is being exploited at our center in its current phase I trial of continuous infusion HMBA.

Measurement of plasma concentrations of HMBA metabolites should also allow more rational in vitro studies of this agent. Knowledge of pharmacologically relevant concentrations of metabolites is allowing us to screen these compounds individually for differentiating activity and to combine them with HMBA in mixtures of representative concentrations to examine their impact on the differentiating activity of HMBA.

The last group of studies utilizing the methodology described in this paper are examining the potential metabolism of HMBA by various malignant cells and the relationship of that metabolism to drug activity.

In summary, we have developed a facile GC analysis that allows quantification of four recently identified metabolites of the cell differentiating agent HMBA. This methodology has the potential for making a major impact on both clinical and preclinical studies of HMBA.

ACKNOWLEDGEMENTS

The authors thank H.C. Spiker and B. Knickman for excellent secretarial assistance in prepartion of the manuscript. This work was supported in part by Contract No. NO1-CM-47734, awarded by the National Cancer Institute, DHHS

and American Cancer Institutional Research Grant IN-174D, Maryland Cancer Program, University of Maryland.

REFERENCES

1 R.C. Reuben, R.L. Wife, R. Breslow, R.A. Rifkind and P.A. Marks, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 862-866.
2 M. Tanaka, J. Levy, M. Terada, R. Breslow, R.A. Rifkind and P.A. Marks, Proc. Natl. Acad. Sci. U.S.A., 72 (1975) 1003-1006.
3 R.C. Reuben, P.L. Khanna, Y. Gazitt, R. Breslow, R.A. Rifkind and P.A. Marks, J. Biol. Chem., 253 (1978) 4214-4218.
4 R.C. Reuben, Biochim. Biophys. Acta, 588 (1979) 310-321.
5 R.C. Reuben, R.A. Rifkind and P.A. Marks, Biochim. Biophys. Acta, 605 (1980) 325-346.
6 E. Fibach, R.C. Reuben, R.A. Rifkind and P.A. Marks, Cancer Res., 37 (1977) 440-444.
7 Y. Gazitt and C. Friend, Cancer Res., 40 (1980) 1727-1732.
8 S.J. Collins, A. Bodner, R. Ting and R.C. Gallo, Int. J. Cancer, 25 (1980) 213-218.
9 A.S. Rabson, R. Stern, T.S. Tralka, J. Costa and J. Wilczek, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 5060-5064.
10 C. Palfrey, Y. Kimhi, U.Z. Littauer, R.C. Reuben and P.A Marks, Biochem. Biophys. Res. Commun., 76 (1977) 937-942.
11 Y. Kloog, J. Axelrod and I. Spector, J. Neurochem., 40 (1983) 522-529.
12 E.H. Hughes, H.A.J. Schut and S.S. Thorgeirsson, In Vitro, 18 (1982) 157-164.
13 W.C. Speers, C.R. Birdwell and F.J. Dixon, Am. J. Pathol., 97 (1979) 563-584.
14 P.A. Marks and R.A. Rifkind, Cancer, 54 (1984) 2766-2769.
15 M.B. Sporn, A.B. Roberts and J.S. Driscoll, in V.T. Devita, S. Hellman and S.A. Rosenberg (Editors), Cancer: Principles and Practice of Oncology, J.B. Lippincott, Philadelphia, PA, 2nd ed., 1985, pp. 49-65.
16 A. Block, Cancer Treat. Rep., 68 (1984) 199-205.
17 E.N. Spremulli and D.L. Dexter, J. Clin. Oncol., 2 (1984) 227-241.
18 A.C. Sartorelli, Br. J. Cancer, 52 (1985) 293-302.
19 P. Calabresi, D.L. Dexter and G.H. Heppner, Biochem. Pharmacol., 28 (1979) 1933-1941.
20 American Cancer Society, Ca., 33 (1983) 122-125.
21 K. Tauer, N. Kemeny and P. Hollander, Proc. Ann. Meet. Am. Assoc. Cancer Res., 26 (1985) 169.
22 A. Yagoda, C. Sternberg, H. Scher and P. Hollander, Proc. Am. Soc. Clin. Oncol., 4 (1985) 105.
23 P.J. O'Dwyer, M. Donehower, L.M. Sigman, C.F. Fortner, J. Aisner and D.A. Van Echo, J. Clin. Oncol., 3 (1985) 853-857.
24 J.G. McVie, W.W. ten Bokkel Huinink, G. Simonetti and R. Dubbelman, Cancer Treat. Rep., 68 (1984) 607-610.
25 D.S. Ettinger, D.W. Orr, A.P. Rice and R.C. Donehower, Cancer Treat. Rep., 69 (1985) 489-493.
26 D.W. Orr, D.S. Ettinger, A.P. Rice, O.M. Colvin, L.B Grochow and R.C. Donehower, Proc. Am. Soc. Clin. Oncol., 2 (1983) 24-93.
27 M.C. Wiemann, F.J. Cummings, M.R. Posner, J.H. Weens, G.W. Crabtree, B.K. Birmingham, A. Moore and P. Calabresi, Proc. Am. Soc. Clin. Oncol., 4 (1985) 38.
28 E.K. Rowinsky, D.S. Ettinger, R.C. Donehower, L.B. Grochow, R.B. Brundrett, A.E. Cates and A.P. Rice, Proc. Am. Soc. Clin. Oncol., 5 (1986) 31.
29 M. Egorin, M. Whitacre, D.A. Van Echo, L. Sigman, A. Forrest, B. Nayar and P. Callery, Proc. Am. Assoc. Cancer Res., 27 (1986) 170.
30 L.M. Sigman, D.A. Van Echo, M.J. Egorin, M.Y. Whitacre and J. Aisner, Proc. Am. Soc. Clin. Oncol., 5 (1986) 34.
31 P.S. Callery, M.J. Egorin, L.A. Geelhaar and M.S.B. Nayar, Cancer Res., 46 (1986) 4900-4903.
32 C.W. Tabor, H. Tabor and U. Bachrach, J. Biol. Chem., 239 (1964) 2194-2203.
33 J.A. Kelley, J.S. Roth and C.L. Litterst, Anal. Lett., 18 (1985) 1043-1062.
34 H. Chun, B. Leyland-Jones, D. Hoth, D. Shoemaker, M. Wolpert-Defillipes, C. Grieshaber, J. Cradock, P. Davignon, R. Moon, R. Rifkind and R. Wittes, Cancer Treat. Rep., 70 (1986) 991-996.