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Simultaneous measurement of the cell-differentiating agent hexamethylene bisacetamide and its metabolites by gas chromatography

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Abstract

Hexamethylene bisacetamide (HMBA) is a potent *in vitro* differentiating agent that has clinical potential as an anticancer drug both as a single agent and as a component of combination therapy. A sensitive and efficient GC method for the isolation, derivatization, and measurement of both HMBA and its two major metabolites in plasma and urine in a single analysis is described. *In situ* carbamylation of the biological sample with diethylpyrocarbonate forms the urethane derivative of the basic N-acetyl diaminohexane metabolite and allows analyte isolation and concentration by solid-phase extraction. Subsequent formation of the *n*-butyl ester of 6-acetamidohexanoic acid, the major metabolite, provides a derivatized biological extract that can be rapidly analyzed by temperature-programmed GC. The quantitative extraction and the efficient derivatization steps provide a limit of quantitation of 0.05 m*M* (10 μg/ml) for all analytes with a precision better than 8% for the range of *in vitro* activity (0.1–2.0 m*M*). This method is amenable to automation and is well-suited for the analysis of clinical samples.

1. Introduction

Hexamethylene bisacetamide (HMBA, NSC 95580, Fig. 1) is an experimental anti-cancer agent that is a potent differentiating agent *in vitro* for many rodent and several human tumor cell lines [1–3]. This *in vitro* differentiating activity depends on both drug concentration (5 m*M* optimum) and duration of drug exposure (5–7 days required). HMBA represents a novel approach to cancer chemotherapy in that neoplastic cells are induced to normalcy rather than selectively killed by a cytotoxic agent. HMBA has been chosen for clinical evaluation solely on the basis of this activity as a differentiating agent [4]. Although more potent analogues in this class

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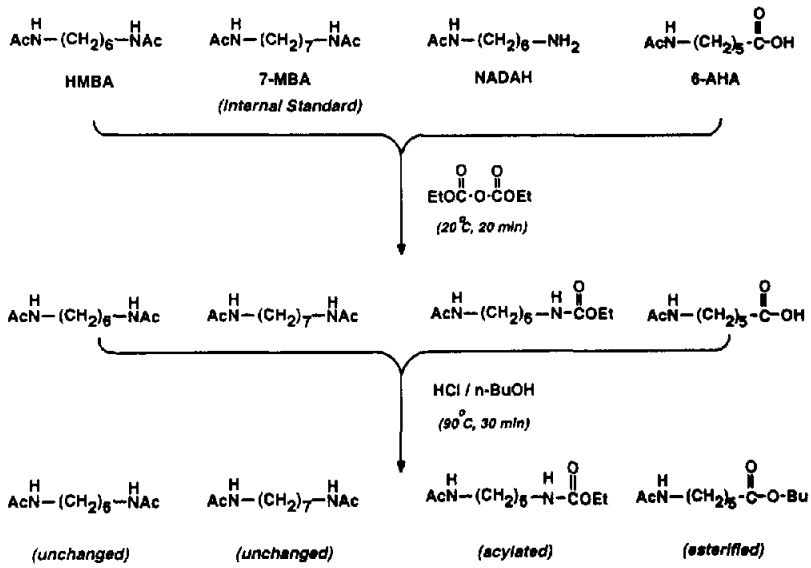

Fig. 1. Structures of HMBA, the 7-MBA internal standard, and the two major metabolites, 6-AHA and NADAH. The *in situ* derivatization scheme for 6-AHA and NADAH in plasma ultrafiltrate or diluted urine is outlined.

of polar-planar compounds have recently been identified [5], HMBA is one of the few for which it has been documented that plasma levels necessary for *in vitro* activity can be maintained *in vivo* [6]. HMBA has undergone Phase I testing on both prolonged continuous infusion schedules [7–11] and on an oral dosing schedule involving both liquid [12] and tablet administration [13]. This new drug is currently being evaluated in Phase II clinical trials sponsored by the National Cancer Institute (NCI) [14].

HMBA is extensively metabolized *in vivo* to both acidic and basic metabolites [15,16]. The two major plasma metabolites, which together with the parent drug also account for the vast majority of urinary excretion, are 6-acetamidohexanoic acid (6-AHA, Fig. 1) and N-acetyl-1,6-diaminohexane (NADAH, Fig. 1) [17]. Plasma concentrations of these two metabolites may be clinically significant to the overall spectrum of activity and toxicity observed in Phase I clinical trials. The major metabolite, 6-AHA, acts synergistically with HMBA to induce differentiation in human promyelocytic leukemia cells *in vitro* [18] and may be partially responsible for the anion gap metabolic acidosis observed at higher doses of drug [9]. NADAH has independent differentiating activity in this same *in vitro* system and an effect that is additive to that of HMBA [18].

Several gas chromatographic methods [8,19,20], as well an HPLC procedure [21], have been reported for the measurement of HMBA in biological fluids. In addition, a GC procedure requiring separate derivatizations has been described for determining the various HMBA metabolites [22]. This latter assay, however, requires two separate sample treatment procedures and two different analysis conditions to measure the HMBA metabolites and does not allow quantitation of parent drug. In order to study the metabolism and disposition of HMBA in patients more easily, we have extended our original method for measuring only HMBA to permit simultaneous determination of HMBA and its major acidic (6-AHA) and basic (NADAH) metabolites. Derivatization in the aqueous phase has been used to render the polar HMBA metabolites suitable for isolation and concentration by solid-phase extraction so that detection of both HMBA and its metabolites is enhanced. The derivatization scheme has also been chosen so that only a single temperature-programmed analysis is required to measure

HMBA and its major metabolites. This method thus allows rapid measurement of HMBA and its bioactive metabolites in patients so that it is useful both for pharmacokinetic studies and for clinical schemes involving adaptive control of drug dosing [11,23,24].

This report details the derivatization, isolation and chromatographic procedures to measure HMBA, 6-AHA and NADAH in biological fluids with one analysis. This method can be partially automated and is suitable for the rapid and routine analysis of large numbers of clinical samples. Both the oral bioavailability of HMBA in humans [12] as well as the comparative drug metabolism after oral and i.v. administration [13] have been determined with this procedure.

2. Experimental

2.1. Materials

HMBA was of pharmaceutical purity and was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI. Heptamethylene bisacetamide (7-MBA) was used as the internal standard and was synthesized as previously described [19]. 6-Acetamidohexanoic acid, 1,6-diaminohexane and 6-aminohexanoic acid were purchased from Aldrich (Milwaukee, WI, USA), while diethyl pyrocarbonate was obtained from Fluka (Ronkonkoma, NY, USA). N-acetyl-1,6-diaminohexane was a generous gift from Dr. Patrick S. Callery, Department of Medicinal Chemistry/Pharmacognosy, School of Pharmacy, University of Maryland, Baltimore, MD, USA [15]. HCl/*n*-butanol (approximately 3 *M* in HCl) was prepared by adding 20% (by volume) concentrated HCl dropwise to *n*-butanol. This reagent was stable for one month when stored at room temperature. Individual 20–25 m*M* standard solutions of HMBA and its metabolites (6-AHA and NADAH) were prepared in distilled water, while the concentration of the 7-MBA internal standard solution was 18.4 m*M* (3.93 mg/ml). These aqueous solutions were stable indefinitely when stored tightly sealed at 4°C. All other chemicals and solvents were reagent grade and were used without further purification.

2.2. Apparatus

Analyses were performed on a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) that was maintained at 275°C and operated with an air flow-rate of 390 ml/min and a hydrogen flow-rate of 40 ml/min. A 1.83 m × 2 mm I.D. glass column, packed with 3% SP2250 DB on Supelcoport 100/120 mesh (Supelco, Bellefonte, PA, USA), was initially held at 200°C for 3 min before being temperature-programmed at 6°/min to a final holding temperature of 236°C. The column was packed so that a standard 10-μl syringe with a 2-inch needle would just touch the packing material when it was fully inserted into the injector, which was maintained at 260°C. Helium was used as a carrier gas at a flow-rate of 30 ml/min, and 2.0-μl aliquots of methanol sample solutions were either injected manually or via a Hewlett-Packard 7673A autosampler. Peak areas were integrated using a Spectra-Physics Chromstation AT chromatography data system (Spectra-Physics, San Jose, CA, USA) consisting of an SP4200 computing integrator connected to a Compaq 386 Model 70 microcomputer (Compaq, Houston, TX, USA). Automatic operation was effected by a remote start signal from the autosampler to the GC and integrator.

Electron ionization mass spectra were obtained on a GC–MS system consisting of a Hewlett-Packard 5710A gas chromatograph interfaced to a VG 7070E mass spectrometer (VG Analytical, Manchester, UK), which was operated under the control of a VG 11-250J+ data system. Spectra were acquired between *m/z* 20 and *m/z* 600 by repetitive scanning at a rate of 1 s/decade during GC analysis of a mixture of derivatized standards under the conditions listed above. The following mass spectrometer operating conditions were used: accelerating voltage, 6kV; ionizing energy, 70 eV; source temperature,

220°C; jet separator, 250°C. Background was automatically subtracted from all mass spectra.

2.3. Biological samples

Plasma and aliquots of 24-h urine collections were obtained from patients treated under a Phase I clinical protocol for oral HMBA that was conducted at Walter Reed Army Medical Center (WRAMC) [12,13]. This study was approved by the WRAMC Institutional Review Board, and all participants enrolled into this clinical trial voluntarily agreed to participate and gave written informed consent. Both plasma and urine were stored frozen at −20°C until analysis.

2.4. Sample preparation

The isolation and derivatization of HMBA and its metabolites in plasma and urine is outlined in Fig. 1. 7-MBA internal standard (20 μl) is added to a 0.4-ml aliquot of plasma, which is diluted and adjusted to a pH greater than 9 with 0.8 ml of 0.1 *M* ammonium hydroxide. This plasma sample is vortex-mixed and then ultrafiltered by centrifuging at 2000 *g* for 25 min in an Amicon Centrifree micropartition unit (Amicon, Bedford, MA, USA). For urine, 10 μl of internal standard solution is added to a 0.2-ml aliquot of 1:100 diluted urine and the pH adjusted with 0.2 ml of ammonium hydroxide. An appropriate aliquot of ultrafiltered plasma or the entire pH-adjusted urine sample is then derivatized by reaction with 10 μl of diethyl pyrocarbonate at room temperature for 20 min. The derivatized sample is adjusted to pH 3 with 0.1 *M* HCl (0.16 ml for urine or 0.3 ml for plasma), and the top half is loaded onto an activated Baker C_{18} solid-phase extraction cartridge (J.T. Baker, Phillipsburg, NJ, USA). The cartridge is then rinsed with 2 ml of 0.1 *M* acetic acid using a Baker-10 SPE vacuum manifold. Compounds of interest are manually eluted with 1.0 ml of methanol, which is collected in a 2-ml conical vial and evaporated to dryness under nitrogen at 40°C. The resultant residue is vortex-mixed with 0.5 ml of 20% (v/v) concentrated HCl–*n*-butanol and heated in a sand bath at 90°C for 30 min. The esterification reagents are evaporated to dryness under nitrogen at 50°C and the final residue is dissolved in 50 μl of methanol prior to GC analysis.

2.5. Standard curves

Standard curves were generated for each patient individually by spiking 0.4-ml aliquots of pretreatment (*i.e.* blank) plasma with HMBA, 6-AHA and NADAH to cover the expected *in vivo* concentration range. Standard solutions of the three analytes were used directly for spiking or diluted 1:10 for the lower concentration ranges. Calibration curves for urine were generated in a similar manner, except that 0.2-ml aliquots of pretreatment urine diluted 1:100 were spiked. Spiked plasma and urine samples were processed and analyzed as described above. Standard curves were established by linear least-squares regression analysis of the measured peak-area ratio of analyte to internal standard *versus* the analyte concentrations added to plasma. Plasma concentrations of HMBA, 6-AHA or NADAH in unknown samples were calculated from the measured peak-area ratio using the appropriate standard curve.

2.6. Recovery, precision and accuracy

The recovery of HMBA, 6-AHA and NADAH from plasma was determined for concentrations of 25, 100 and 400 μg/ml and from urine for a concentration of 100 μg/ml. The recovery of the 7-MBA internal standard from plasma and urine was also measured for a level of 200 μg/ml, the concentration normally used for biological samples. Aliquots of pretreatment plasma (2.0 ml) or urine (1.0 ml) were spiked to the above concentrations with concentrated aqueous standards, and four aliquots were taken from each individual solution to be processed as described above. In addition, unspiked plasma and urine were treated in the same manner. The peak areas of HMBA and 7-MBA in spiked samples were compared directly to those of standards of the same concentration in methanol. Since pure standards of the 6-AHA and

NADAH derivatives were not available, their peak areas in spiked samples were compared to those of appropriate minimally processed samples. For 6-AHA, methanol solutions were evaporated, reacted with HCl–*n*-butanol, evaporated and reconstituted in 50 μl of methanol. For NADAH, aqueous solutions were added to 0.1 *M* ammonium hydroxide, reacted with diethyl pyrocarbonate, evaporated with the aid of methanol as a cosolvent and reconstituted in 50 μl of methanol.

Standards were analyzed after every two spiked samples and sample peak areas were compared to the closest corresponding standard peak area. Recovery was the mean of these comparisons expressed as a percentage. The relative standard deviation of the ratios of the peak area of the compound of interest to that of the internal standard was used as a measure of assay precision.

Assay accuracy was determined for each analyte using a series of spiked plasma samples of known concentration covering the range of the appropriate standard curve. The accuracy for an individual analyte was defined as the mean of the absolute percent difference between the measured concentration and the known concentration of this analyte in each sample of the series.

3. Results and discussion

Of the six HMBA metabolites which have been identified in humans [15,16], 6-AHA and NADAH are by far the most prevalent [12,15,17,22]. Both metabolites are found in the plasma and urine of cancer patients treated with HMBA and, when combined with unchanged drug, account for the vast majority of the administered dose that is excreted in the urine (*vide infra*) [12,17]. The other metabolites have not been detected in the plasma of patients receiving up to 43 g/m^2/day, and their urinary excretion accounts for less than 3% of the daily administered dose of HMBA. Thus analysis of biological fluids for the above two major metabolites and HMBA itself essentially defines the disposition of this drug in humans.

Analysis of the 6-AHA and NADAH metabolites requires derivatization both to facilitate isolation by solid-phase extraction and to permit separation by gas chromatography. Since solid-phase extraction functions best for aqueous solutions of lipophilic analytes, a derivatization scheme that converts polar functional groups to more lipophilic moieties and can be carried out *in situ* in the biological sample is desirable. Also, since 6-AHA is acidic and NADAH is basic, at least one or both have to be rendered neutral before solid-phase extraction with a C_{18} cartridge can be employed efficiently. Schotten–Baumann acylation of a basic amino moiety is a well-known transformation that can be carried out under basic conditions in aqueous media [25]. Several derivatization schemes of this type, as well as others that can be carried out in an aqueous matrix [26], have been evaluated, with carbamylation using diethyl pyrocarbonate (ethoxyformic acid anhydride) [27] being the most suitable for this analysis. The urethane derivative of NADAH (Fig. 1) is formed quantitatively within 20 min at room temperature with this reagent, when the plasma ultrafiltrate or diluted urine is adjusted to pH 9 or higher. These reaction conditions are also mild enough that no decomposition of HMBA or its 7-MBA homologue, which is employed as an internal standard, is observed. Once derivatization of NADAH has occurred, the pH of the plasma ultrafiltrate or urine is adjusted to 3 so that the carboxylic acid of 6-AHA is no longer ionized. At this stage, solid-phase extraction can be employed to isolate and concentrate both HMBA and its metabolites. The compounds of interest are eluted from the C_{18} cartridge with methanol, which can be easily evaporated to give a sample amenable to derivatization of 6-AHA. Fischer esterification of this carboxylic acid to form a butyl ester gives a derivative with good chromatographic properties [28,29]. These derivatized and processed plasma and urine samples and spiked standards are stable for at least one week if stored at 4°C.

The two-step derivatization scheme of Fig. 1 is also capable of derivatizing 1,6-diaminohexane and 6-aminohexanoic acid, two minor metabo-

lites of HMBA. GC–MS analysis of a mixture of standards confirmed that the expected derivatives of these and the two major metabolites are formed (Table 1). All derivatives are well separated from each other and from HMBA and 7-MBA on a moderately polar packed column (Table 1, Kovats retention index [30]). Tailing is minimized by using a base-deactivated solid support, and temperature programming allows rapid analysis with a 20-min cycle time (Fig. 2). This derivatization scheme and the above chromatographic conditions also produce minimal interference at the expected retention times of 6-AHA, NADAH or HMBA when either blank plasma (Fig. 2A) or blank urine (Fig. 3A) is analyzed. Furthermore, injection of the sample as a methanol solution overcomes the problems of column deterioration noted previously with the direct injection of plasma ultrafiltrate and diluted urine [19,20].

The recovery of analytes from spiked plasma and urine was measured by direct comparison of absolute chromatographic peak areas (Table 2). HMBA and 7-MBA were evaluated directly against standards, and their recovery reflects both solid-phase extraction isolation efficiency and any decomposition occurring during derivatization. Recovery of these two compounds was essentially quantitative and equivalent to that obtained with solid-phase extraction alone. Standards were not available for 6-AHA butyl ester or NADAH urethane, so recovery was evaluated against derivatized standards of the metabolites themselves. For NADAH, recovery reflects isolation efficiency and any decomposition during esterification. For 6-AHA, recovery measures the isolation efficiency of free underivatized acid, since all the other parameters are equal.

Standard curves for HMBA and the 6-AHA and NADAH metabolites are generated for each individual patient from 5–7 aliquots of pretreated plasma and urine spiked with different and varying proportions of the three analytes (Table 3). That is, an individual spiked plasma or urine standard might contain a high concentration of HMBA, an intermediate level of 6-AHA and a small amount of NADAH. These standard curves exhibit the best linearity when they are based on area ratios of analyte to internal standard (Table 3). In plasma, this linearity coincides with the clinically relevant range of 10–500 μg/ml for all three analytes with a corresponding limit of quantitation ($S/N \geq 5$)

Table 1
Analytical characteristics of HMBA and its metabolites

Compound	Derivative	Retention index[a]	M_r	Electron ionization mass spectrum[b] (m/z)
HMBA	–	2557	200	200 (2.8), 185 (1.2), 157 (6.3), 128 (43), 86 (34), 72 (42), 43 (59), 30 (100)
7-MBA	–	2658	214	214 (3.1), 199 (1.1), 171 (6.6), 142 (31), 86 (27), 72 (38), 43 (54), 30 (100)
6-AHA	*n*-Butyl ester	2200	229	229 (5.6), 186 (24), 156 (5.4), 114 (100), 72 (40), 60 (26), 43 (49), 30 (92)
NADAH	Ethoxycarbamate	2465	230	230 (1.9), 187 (3.9), 185 (4.1), 158 (8.8), 86 (18), 72 (28), 43 (40), 30 (100)
6-AmHA[c]	Ethoxycarbamate, *n*-butyl ester	2151	259	259 (2.1), 214 (2.8), 186 (48), 140 (58), 102 (78), 56 (70), 30 (100), 29 (85)
DAH[d]	Bis(ethoxycarbamate)	2392	260	260 (5.0), 215 (10), 158 (24), 141 (21), 130 (58), 102 (96), 74 (45), 30 (100)

[a]Kovats retention indices determined isothermally at either 190° or 220°C.
[b]Mass spectra are the weighted average of 3–5 contiguous repetitive scans obtained by GC–MS. Values in parentheses are the relative intensities in %.
[c]6-Aminohexanoic acid.
[d]1,6-Diaminohexane.

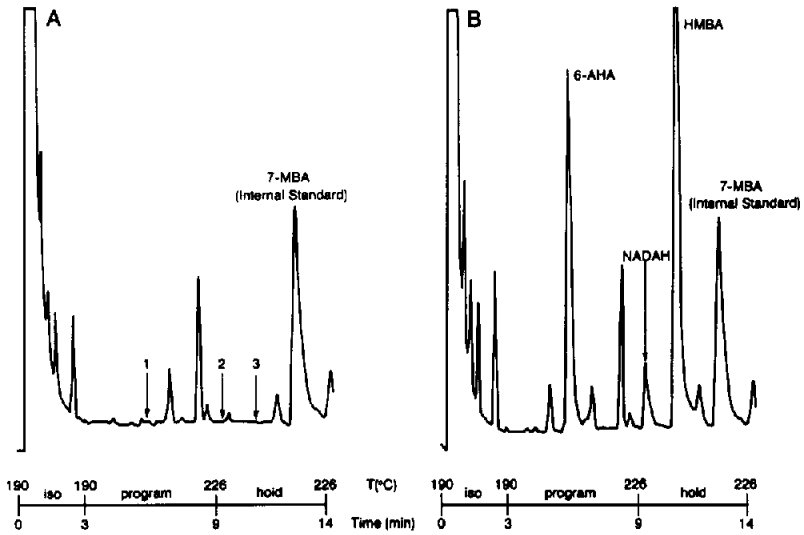

Fig. 2. Temperature-programmed gas chromatogram of the derivatized isolate from (A) pretreatment patient plasma, and (B) patient plasma 116 h after initiation of a 5-day regimen of 30 g/m^2/day HMBA administered orally via nasogastric tube in 30 equal doses of 5 mg/m^2 every 4 h. In chromatogram A, the numbered arrows indicate the expected retention times of 6-AHA (1), NADAH (2) and HMBA (3). In chromatogram B, measured concentrations are: 6-AHA, 148 μg/ml; NADAH, 47 μg/ml; and HMBA, 448 μg/ml (2.24 mM). 7-MBA has been added to all plasma samples as an internal standard at a concentration of 196 μg/ml.

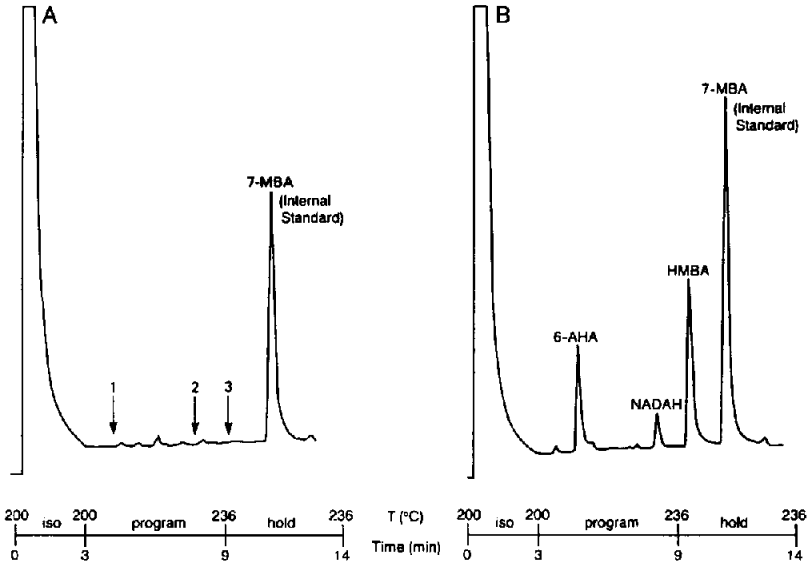

Fig. 3. Temperature-programmed gas chromatogram of the derivatized isolate from (A) diluted (× 100) pretreatment patient urine and (B) a diluted (× 100) 24-hr collection from day 3 of a patient treated with 30 g/m^2/day HMBA as a 5-day continuous infusion. In chromatogram A, the numbered arrows indicate the expected retention times of 6-AHA (1), NADAH (2) and HMBA (3). In chromatogram B, measured concentrations are: 6-AHA, 41 μg/ml; NADAH, 25 μg/ml; and HMBA, 99 μg/ml. 7-MBA has been added to all diluted urine samples as an internal standard at a concentration of 200 μg/ml.

Table 2
Analytical recovery and assay precision using spiked human plasma and urine

Concentration (μg/ml)		Recovery[a] (%)				Precision[c] (R.S.D., %)		
		6-AHA	NADAH	HMBA	7-MBA[b]	6-AHA	NADAH	HMBA
Plasma	25	100	92	105	99	4.5 (0.138)	3.6 (0.075)	4.4 (0.111)
	100	96	101	110	112	7.9 (0.517)	6.2 (0.305)	4.0 (0.433)
	400	109	111	116	98	2.5 (2.36)	1.6 (1.52)	1.5 (2.19)
Urine	100	88	97	88	88	0.9 (0.537)	3.9 (0.404)	1.0 (0.448)

[a] Mean of four independent workup procedures and analyses.
[b] Concentration was 200 μg/ml in all cases.
[c] Determined from peak-area ratio of analyte to internal standard (7-MBA). Mean ratio ($n = 4$) indicated in parentheses.

of 10 μg/ml (0.05 m*M* for HMBA). A non-zero intercept is apparent for the 6-AHA methyl ester (Table 3), and this reflects the low level (10–15 μg/ml) interference present in plasma at this retention time (Fig. 2A). The difference in slope observed for the three analytes of Table 3 is probably partly due to differences in relative detector response and, in the case of the NADAH, reproducible but non-quantitative derivatization efficiency. Assay precision, which is expressed as the relative standard deviation of peak-area ratios, is better than 8% at all levels for all three analytes (Table 2). Assay accuracy for each analyte over the range 10–400 μg/ml

Table 3
Standard curves for HMBA and metabolites in patient plasma and urine[a]

Parameter	HMBA	6-AHA	NADAH
Plasma			
Slope	0.00515	0.00773	0.00377
S.E.[b] of slope	0.000088	0.000169	0.000064
Intercept	0.0385	0.1027	0.0279
S.E. of intercept	0.0196	0.0346	0.00695
Range (μg/ml)[c]	10–512	28–435	12–249
Number of standards[d]	7	6	7
Correlation coefficient (r)	0.9993	0.9991	0.9993
Urine			
Slope	0.00486	0.00620	0.00332
S.E. of slope	0.000060	0.000037	0.000017
Intercept	−0.0166	−0.00531	0.0165
S.E. of intercept	0.0133	0.00708	0.00104
Range (μg/ml)	10–512	11–435	12–125
Number of standards	7	7	6
Correlation coefficient (r)	0.9996	0.9999	0.9999

[a] Plasma and urine were obtained from the same patient.
[b] Standard error.
[c] Concentration range over which samples were spiked.
[d] Includes blank or zero point.

averages better than 9% with that for HMBA being 5.5% ($n = 5$), 6-AHA being 8.6% ($n = 6$), and NADAH being 3.7% ($n = 6$).

The GC method described here has been used successfully to analyze clinical samples from an extensive multi-faceted Phase I study [12,13,31]. Plasma concentrations of HMBA and its major metabolites have been compared following administration of identical drug doses both by prolonged i.v. infusion (Fig. 4A) and by repetitive oral administration via a nasogastric tube (Fig. 4B) in the same patient. Plasma levels and post-infusion plasma elimination of HMBA, 6-AHA and NADAH are comparable with either route of administration. As can also be seen in Fig. 4, plasma concentrations of HMBA (1–5 m*M*), 6-AHA (>0.5 m*M*) and NADAH (>0.1 m*M*) that correspond to those required for *in vitro* differentiating activity are also achieved in patients. Urinary excretion of parent drug and metabolites after the different 5-day dosing regimens has also been measured. Fig. 5, which depicts the cumulative elimination of HMBA, 6-AHA and NADAH in the urine for a patient given oral HMBA via nasogastric tube, indicates that the vast majority of the dose is accounted for by these three compounds. In addition, the disposition of HMBA is the same regardless of the mode of administration. These analyses have been used to show that HMBA has essentially complete (>97%) oral bioavailability whether administered as a solution via nasogastric tube [12] or as a compressed solid in 1-g tablets [13,31]. Full details of these studies are reported in the indicated references [12,31].

The method described above is well suited for the analysis of the large numbers of samples likely to be encountered in a clinical or a pharmacokinetic study. Samples and standards are typically prepared as a batch procedure, and the analysis is carried out using an autosampler for automated injection. This level of automation is straightforward since only one workup procedure and a single set of chromatography conditions are required to measure HMBA and its two major metabolites simultaneously. Since

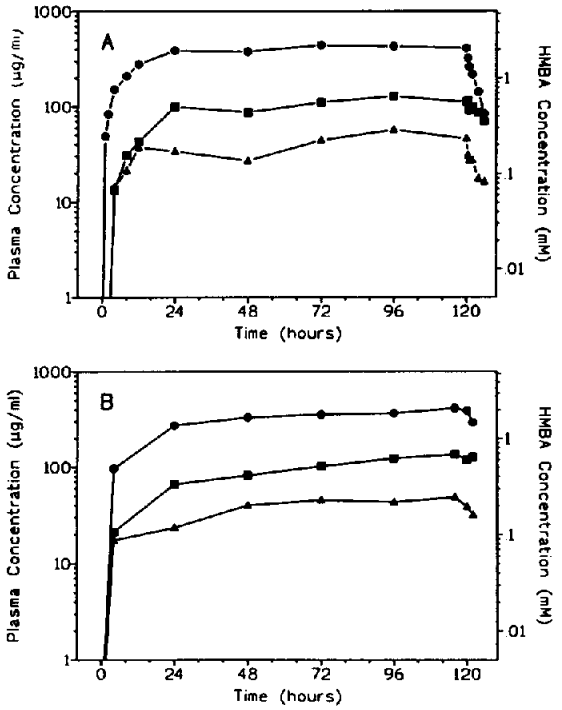

Fig. 4. Plasma concentration *versus* time curve for the same patient during and following a 5-day treatment with 30 g/m^2/day HMBA as (A) a continuous infusion and (B) an oral dose via nasogastric tube (5 g/m^2 every 4 h). Plasma samples during oral dosing were obtained 4 h after administration of drug via nasogastric tube. Thus, plasma concentrations in (B) represent the minimum levels attained during repetitive dosing. The bioavailability of HMBA admimistered orally via nasogastric tube was 98%. Key: HMBA (●); 6-AHA (■); NADAH (▲).

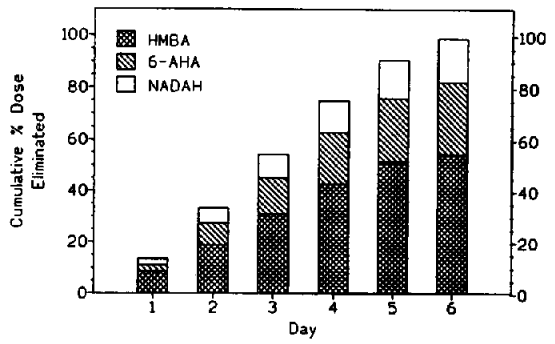

Fig. 5. Cumulative urinary excretion of HMBA and its two major metabolites (6-AHA and NADAH) in a cancer patient expressed as a percentage of the administered HMBA dose. A 5-day dose of 36 g/m^2/day HMBA was administered via nasogastric tube. Urinary excretion accounted for 99% of the administered dose.

the derivatives are stable in methanol solution for up to a week if refrigerated, analysis does not have to be performed immediately after sample processing. Therefore, samples may be accumulated for more efficient GC analysis. If necessary, a further level of automation could be incorporated through the use of a robotic sample preparation system for all or some of the derivatization and solid-phase extraction.

This GC method possesses comparable sensitivity and linearity for 6-AHA and NADAH to that of a previously reported method for the measurement of HMBA metabolites only [22], with the added advantage of measuring HMBA and using only one internal standard and one set of chromatographic conditions. Recent *in vitro* studies have shown that combination of HMBA with other differentiating agents potentiates activity in a synergistic manner [32–35]. If the clinical potential of these combination therapies can be realized, the lower target plasma concentrations required for activity should result in reduced doses and hopefully less toxicity than observed during the single-agent clinical trials. The described GC method should also be ideally suited to evaluate the pharmacokinetics, metabolism and disposition of HMBA in combination therapies in future preclinical and clinical studies. Although an FID has been adequate for the analysis of clinical samples encountered in this study, any additional selectivity and sensitivity required with combination therapy might be achieved with the use of a nitrogen–phosphorus detector, as in our initial analysis of HMBA [19] and by others using alternative methods [20,22]. Capillary GC with wide-bore columns [20] should also be easily adapted to this isolation and derivatization scheme to increase sensitivity through improved peak shape and better resolution of trace components.

4. Conclusion

A comprehensive GC method has been developed and evaluated for the simultaneous measurement of HMBA and its two major metabolites, 6-AHA and NADAH, in biological fluids. This method has been employed to determine the plasma pharmacokinetics and metabolism, urinary disposition, and bioavailability of HMBA in a multi-faceted Phase I clinical trial involving i.v. and oral administration. The described method is suitable for drug level monitoring during extended HMBA therapy using adaptive control of dosing algorithms to control steady-state plasma levels [11]. When used in this manner, a better assessment of differentiating potential should be obtained since both parent drug and major bioactive metabolites are measured. Because this assay is efficient, sensitive and adaptable to automation, it will also be useful for the continued clinical development of HMBA as both a single anticancer agent and as a component of combination therapy.

5. Acknowledgement

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6. References

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