Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2008 February 1; 862(1-2): 168–174.

Liquid chromatography-mass spectrometric assay for quantitation of the short-chain fatty acid, 2,2-dimethylbutyrate (NSC 741804), in rat plasma

Robert A. Parise^{a,b}, Jan H. Beumer^{a,b}, Cyrus O. Kangani^c, Julianne L. Holleran^a, Julie L. Eiseman^{a,d}, Nicola F. Smith^e, Joseph M. Covey^e, Susan P. Perrine^f, and Merrill J. Egorin^{a,d,g}

^a Molecular Therapeutics/Drug Discovery Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA

^b Department of Pharmaceutical Sciences, University of Pittsburgh School of Pharmacy, Pittsburgh, PA, 15213, USA

^c Division of Endocrinology and Metabolism, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

^d Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

^e Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Rockville, MD 20852, USA

^f Cancer Research Center, Boston University School of Medicine, Boston, MA 02118, USA

^g Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

Abstract

2,2-Dimethylbutyrate (DMB) is a potential treatment for thalassemia and hemoglobinopathies. To facilitate pharmacokinetic evaluation of DMB, we developed an LC-MS assay and quantitated DMB in plasma of rats after an oral dose of 500 mg/kg. After acetonitrile protein precipitation, DMB and dimethylvaleric acid (DMV) internal standard were derivatized to benzylamides, chromatographed on a Hydro-RP column with acetonitrile, water, and 0.1% formic acid, and detected by electrospray positive-mode ionization mass spectrometry. The assay was accurate (97–107%) and precise (3.4–6.2%) between 100 and 10,000 ng/mL. Recovery from plasma was >62%. Plasma freeze-thaw and room temperature stability were acceptable.

Keywords

dimethylbutyrate; metabolism; mass spectrometry; validation; fatty acid; sickle cell disease; thalassemia

Correspondence to: Merrill J. Egorin M.D., University of Pittsburgh Cancer Institute, Room G27E, Hillman Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213-1863, USA, Tel.: +1-412-623-3252, Fax.: +1-412-623-1212, Email: egorinmj@msx.upmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

β -thalassemia and sickle cell disease are caused by gene mutations affecting the β -globin chain of adult hemoglobin A and are associated with severe morbidity and early mortality [1;2]. One approach to treating these diseases is reactivating expression of developmentally silenced fetal globin genes, which can functionally substitute for deficient β -globin in β -thalassemia and inhibit sickling in the sickle syndromes [2–7]. Short-chain fatty acids, such as butyric acid, stimulate fetal globin gene expression in experimental models, including patients' cultured cells, transgenic mice, and baboons [8–16]. In clinical trials, short-chain fatty acids have induced fetal hemoglobin and improved total hemoglobin levels, thereby demonstrating proof-of-concept of such an approach to the treatment of hemoglobinopathies [17–22]. However, short-chain fatty acids are: 1) rapidly metabolized, necessitating prolonged intravenous infusions or large oral doses; and 2) exhibit unwanted anti-proliferative effects on erythroid cells [22]. Development of an orally active, short-chain fatty acid with more favorable pharmacologic properties would be a major advance in the treatment of hemoglobinopathies. 2,2-Dimethylbutyrate (DMB) has such favorable properties; it stimulates fetal globin production, prolongs erythroid cell survival, lacks antiproliferative properties and increases cell proliferation by altering the balance of Bcl-family proteins [14–16]. Because preliminary studies have suggested that DMB has favorable pharmacokinetic properties, such as a longer half-life, and an acceptable safety profile [14–16], it has been selected for clinical development. One of the requirements for clinical development is adequate pharmacokinetic characterization of DMB in preclinical models and, eventually, in patients. Such pharmacokinetic studies require a validated analytical method suitable for quantitation of DMB in relevant biological matrices. Because such a method did not exist and because the preclinical toxicology and toxicokinetic studies upon which subsequent clinical trials are being designed were performed in rats, an LC-MS assay was developed to quantitate DMB in rat plasma.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (Optima grade) was purchased from Fisher Scientific (Fairlawn, NJ, USA). Water was purified using a Q-gard® 1 Gradient Milli-Q system (18.2 M Ω .cm, Millipore, Billerica, MA, USA). Formic acid, 2,2-Dimethylbutyric acid, dichloromethane, benzylamine, N,N-diisopropylethylamine, and (Bis(2-methoxyethyl)-amino) sulfur trifluoride (Deoxy-Fluor) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2, 2-Dimethylvaleric acid (DMV) was purchased from TCI America (Portland, OR, USA). Control rat plasma was obtained from control animals or purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Nitrogen (99.99% pure) was purchased from Valley National Gases, Inc. (Pittsburgh, PA, USA). The sodium salt of DMB (NSC D741804) was supplied by the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA).

2.2. Preparation of calibration standards and quality control samples

Stock solutions of DMB were prepared at 1 mg/ml in acetonitrile and stored at 4 °C in the dark. On assay days, this solution was serially diluted (in steps of 10-fold) with water to obtain the lower calibration working solutions. These calibration working solutions were diluted in rat plasma to produce 200- μ l aliquots of the following DMB concentrations: 100, 300, 1000, 3000, 5000, and 10000 ng/ml.

Quality control (QC) stock solutions were prepared independently from a separate weighing of DMB and stored at 4 °C in the dark. These solutions were diluted in rat plasma to produce 200- μ l aliquots of the following QC samples: QC low (QCL) 200 ng/ml; QC mid (QCM) 1000 ng/ml; and QC high (QCH) 7500 ng/ml. In addition, the QC stock solution was used to prepare

rat plasma samples that contained 100 ng/ml DMB, which was the lower limit of quantitation (LLQ) of the assay.

2.3. Sample preparation

Ten μ l of a 10 μ g/ml solution of DMV (internal standard) in acetonitrile and 1 ml of acetonitrile were added sequentially to each tube of 200 μ l standard, QC, or sample plasma. Samples were vortexed for 1 min at a setting of 10 on a Vortex Genie-2 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at $16,000 \times g$ at room temperature for 6 min. The resulting supernatants were transferred to 12 mm $\times$ 75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen at 37 °C. Dried residues were re-dissolved in 500 μ l of dichloromethane. Following the addition of 10 μ l of 4.975% benzylamine in acetonitrile (v/v) and 10 μ l of 4.95% N,N-diisopropylethylamine in acetonitrile (v/v), samples were stored at -20 °C for 20 min. Ten μ l of 60 mg/ml cooled (4 °C) (Bis(2-methoxyethyl)-amino) sulfur trifluoride (Deoxy-Fluor) were added, and samples were incubated at -20 °C for an additional 20 min. The reaction mixtures were then evaporated to dryness under nitrogen at 37 °C. Samples were reconstituted in 100 μ l of acetonitrile: water (25:75, v/v), transferred to 1.5-ml microcentrifuge tubes, and centrifuged at $16,000 \times g$ for 3 min. The resulting supernatants were transferred to autosampler vials, and 5 μ l were injected into the LC-MS system.

2.4. Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 autosampler and binary pump, a Phenomenex (Torrance, CA, USA) Synergi Hydro-RP (4 μ m, 2 $\times$ 100 mm) column kept at ambient temperature, and a gradient mobile phase. Mobile phase solvent A was 0.1% (v/v) formic acid in acetonitrile, and mobile phase solvent B was 0.1 % (v/v) formic acid in water. The initial mobile phase composition of 20% solvent A and 80% solvent B was maintained for 4 min at a flow rate of 0.2 ml/min. Between 4 and 13 min, the percentage of solvent A was increased linearly to 50% and was maintained at 50% until 19 min. Between 19 and 20 min, the percentage of solvent A was increased linearly to 100%, and the flow rate was increased to 0.4 ml/min. These conditions were maintained for 2 min. Between 22 and 23 min, the percentage of solvent A was decreased linearly to 20%. Between 23 min and the end of the run at 30 min, the percentage of solvent A was maintained at 20%.

2.5. Mass spectrometry

Mass detection was carried out using a ThermoFinnigan (San Jose, CA, USA) MSQ single quadrupole mass spectrometer with electrospray ionization in positive-ion mode. For both full-scan and single-ion monitoring (SIM) modes, the settings of the mass spectrometer were as follows: capillary voltage 4.0 kV; cone voltage 60 V; and probe temperature 400 °C. In SIM mode, the m/z -values monitored were 206 and 220 for DMB and DMV, respectively. The dwell time was 0.37 s, and the span was set at 0.2 amu. The LC system and mass spectrometer were controlled by ThermoFinnigan Excalibur software (version 4.0), and data were collected with the same software. The analyte-to-internal standard ratio (response) was calculated for each standard by dividing the area of the analyte peak by the area of the internal standard peak. Standard curves of DMB were constructed by plotting the analyte-to-internal standard ratio *versus* the known concentration of DMB in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, followed by the back calculation of concentrations.

2.6. Assay Validation procedures

2.6.1. Calibration curve and lower limit of quantitation (LLQ)—DMB added to rat plasma and derivatized as described above was injected into the analytical system at decreasing concentrations to determine the minimal concentration with a signal-to-noise ratio of at least

5:1. Calibration standards and blanks were prepared (see section 2.2) and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. Calibration curves were constructed as described above. The deviations of back-calculated concentrations from nominal concentrations, expressed as percentage of the nominal concentration, reflected the assay performance over the concentration range.

2.6.2. Accuracy and precision—The accuracy and precision of the assay were determined by analyzing samples with DMB at the LLQ and QC concentrations in a minimum of 5 replicates in 3 analytical runs together with an independently prepared, triplicate calibration curve. Accuracy was calculated at each test concentration as:

$$(\text{mean measured concentration} / \text{nominal concentration}) \times 100 \%$$

Assay precision was obtained for each test concentration by using the coefficient of variation of the measured concentration calculated from all 15 determinations for inter-assay precision; and calculated from the mean of the three quintuplicate sets for the intra-assay precision.

2.6.3. Selectivity and specificity—To investigate whether endogenous matrix constituents interfered with the assay, four individual batches of control, drug-free rat plasma were processed and analyzed according to the described procedures. Responses of DMB at the LLQ concentration were compared with the response in the blank samples.

2.6.4. Extraction recovery—The recovery of the assay was determined by adding DMB and DMV to the dry residue of 3 blank plasmas that had undergone acetonitrile precipitation and evaporation, and comparing the response to that of samples with DMB and DMV added to 3 fresh plasmas before deproteination and evaporation.

2.6.5. Stability—The stability of a 1 mg/ml DMB stock solution was assessed after storage at 4 °C in the dark for 6 months. The response was compared to a freshly prepared stock solution, and stability was expressed as the percentage recovery of the stored solution relative to the fresh solution. All stability testing in plasma was performed in triplicate at the QCL, QCM, and QCH concentrations. The effect of 3 freeze/thaw cycles on DMB concentrations in plasma was evaluated by assaying samples after freezing and thawing on 3 separate days and comparing them to freshly prepared samples. The stability of DMB in plasma during sample preparation was evaluated by assaying samples before and after 6 h of storage at room temperature. The long-term stability of DMB in plasma was evaluated by assaying samples after 5 months of storage at –80 °C and comparing those results with those of freshly prepared QC samples. To evaluate the stability of DMB in reconstituted samples in the autosampler, we prepared multiple samples of 300 ng/ml DMB in plasma. The samples were extracted, derivatized, evaporated, reconstituted and pooled. The resultant pooled sample was reinjected for 75 runs (37 h).

2.7. Animals and application of the assay

Specific-pathogen-free, adult Fischer 344 male rats were purchased from Hilltop Lab Animals (Scottsdale, PA, USA). Rats were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility for at least 1 week before being used. To minimize infection, rats were maintained in micro-isolator cages in a separate room and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and on a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Ventilation and airflow were set to 12 changes per h. Room temperatures were regulated at 22 ± 1 °C, and rooms were kept on automatic 12-h light/dark cycles. Rats received Prolab ISOPRO RMH 3000 Irradiated Lab Diet (PMI Nutrition International, St. Louis, MO, USA) and water *ad libitum*, except on the evening before dosing, when all food was removed.

Rats were 9–10 weeks old at the time of dosing. Rats were stratified into 5 groups of 3 animals each, and all were fasted overnight prior to dosing. Rats received a single dose of 500 mg/kg DMB sodium salt (equivalent to 414 mg/kg of free DMB) in sterile water (0.005 ml/g body weight) by oral gavage using a 2.5 inch, 18-gauge, ball-tipped oral gavage needle and a 3-cc syringe. Heparinized 1-cc syringes fitted with 25-gauge needles were used to obtain blood samples of approximately 400 μ l from a lateral tail vein of each of the 3 rats per group at the following specified times after DMB administration. For groups 1, 2, 3, 4 and 5, respectively, blood was collected at before and at 10 min after dosing; at 5 and 45 min after dosing; at 15 and 240 min after dosing; at 30 and 90 min after dosing; and at 60 and 180 min after dosing. Groups of 3 rats were euthanized by CO₂ inhalation at 120, 360, 420, 960 and 1440 min after dosing, and each rat was exsanguinated by collection of blood via cardiac puncture into 10-cc heparinized syringes fitted with 20-gauge needles. Heparinized blood was centrifuged at 13,000 $\times$ g at room temperature for 4 min to obtain plasma, which was stored at –80 °C until analysis.

Concentration *versus* time data were analyzed non-compartmentally using the Lagrange function [23] as implemented by the LAGRAN computer program [24].

3. Results and Discussion

3.1. Method development

The development of an analytical method for DMB presented a number of challenges. DMB is a small, very polar molecule, which made its retention on reverse-phase HPLC columns problematic. We initially evaluated columns, such as the Synergi Hydro-RP 2 $\times$ 250 mm and Polar-RP 2 $\times$ 100 mm (Phenomenex, CA, USA), that are designed to retain polar compounds. However, DMB and DMV eluted shortly after the void volume. The use of formic acid, ammonium acetate, and ammonium hydroxide in the mobile phase did not significantly change the retention time of either DMB or DMV. The fact that DMB lacks a chromophore or fluorophore made direct absorbance or fluorescence detection impossible. Mass spectrometric detection was challenging because DMB did not ionize in electrospray positive mode and ionized weakly in electrospray negative mode. Furthermore, the addition of reagents such as formic acid, acetic acid, ammonium acetate and ammonium hydroxide did not alter the ionization characteristics of DMB. Using the Synergi Hydro-RP column for chromatography and electrospray negative ionization for mass spectrometric detection, allowed detection of 1000 ng/ml of neat compound. This was not acceptable for our needs and led us to pursue a derivatization strategy to convert DMB and DMV to their respective amides. The derivatization employed involved modifications to a recently published, mild and highly efficient, one-step procedure that used a deoxo-fluoro reagent [25] to convert DMB and DMV to N-benzyl-2,2-dimethylbutyramide (BDMB) and 2,2-dimethylpentanoic acid benzylamide (BDMV), respectively (Fig. 1). The original derivatization method was developed for GC-MS with electron impact ionization. Through experimentation, we were able to modify the original method without any loss in efficiency when assessed by LC-MS. The first modification involved replacing the NaHCO₃ quenching step and *n*-heptane extraction step, which were included in the original method to remove aqueous material, with direct evaporation of the reaction mixture under a stream of nitrogen at 37 °C. This dried residue was then resuspended in 100 μ l of the initial mobile phase, centrifuged, and 5 μ l of the resulting supernatant were injected into the LC-MS. This shortened sample processing time and increased sensitivity; however, it did result in a more complex sample matrix. To counter this, we examined the effect of reactant concentration on amide production and found that a 20-fold decrease in reactant concentration was tolerated without a decrease in amide production. We also found that 5 μ l was a suitable injection volume, as injection of larger volumes led to a clogged sample cone after <24 h of run time.

3.2 Validation of the assay

3.2.1 Mass spectrometry and chromatography—Approximately 50 mg each of DMB and DMV internal standard were converted to their respective benzyl amides, BDMB and BDMV, as described above and purified by flash chromatography on a silica column with ethyl acetate:heptane (20:80, v/v) as the eluting solvent. The purified compounds were determined to be 99% pure by GC-MS, using an Agilent 6890 gas chromatograph fitted with an HP-5 column (30 m × 0.25 mm i.d., 0.25 µm film thickness) and interfaced with an Agilent 5973N mass spectrometer. The structures of BDMB and BDMV were confirmed by ¹H and ¹³C NMR, using a Varian Mercury 400 spectrometer (Varian Inc., Palo Alto, CA, USA) with spectra recorded at 400 and 100 MHz, respectively. BDMB was diluted in acetonitrile and continuously infused at a rate of 10 µl/min into the HPLC flow, which was an isocratic mobile phase of acetonitrile:water:formic acid (50:50:0.1, v/v/v) pumped at 0.2 ml/min. The tuning parameters of the mass spectrometer were adjusted to maximize the intensity of the [M + H]⁺ ion. The procedure was repeated for BDMV, and the *m/z* ratios of 206 and 220 were chosen for SIM of BDMB and BDMV, respectively. With the chromatographic conditions described, BDMB and BDMV displayed retention times of approximately 16.9 and 18.5 min, respectively (Fig. 2). There was baseline separation of both compounds. To test for interference, 4 different sources of rat plasma were analyzed. None of the sources tested showed interference with DMB or DMV (data not shown).

3.2.2 Linearity—Triplicate standard curves of DMB in plasma were performed on three sequential days. The assay proved to be linear and acceptable, as the regression coefficients were >0.99 for each of the three standard curves with 1/y² weighting (data not shown). The lower limit of quantitation (LLOQ) was determined to be 100 ng/ml, as the signal-to-noise ratio was >5 at this concentration, and there was acceptable accuracy and precision. The individual values for the mean and standard deviations of the back-calculated values at each nominal DMB concentration used in the standard curves are displayed in Table 1, as is the accuracy calculated from those values.

3.2.3 Accuracy and precision—FDA guidelines specify that the accuracies for all tested concentrations should be within ±15%, and the precisions, expressed by the coefficient of variation, should not exceed 15%, except for the LLQ, in which case these parameters should not exceed 20%. The accuracies and intra- and inter-assay precisions for the tested DMB concentrations (LLQ, QCL, QCM, QCH) were all within those acceptance criteria (Table 2).

3.2.4 Extraction recovery—Because the method developed includes a chemical reaction in plasma, ion suppression could not easily be assessed. However, the recovery of the assay was determined by adding DMB and DMV to the residue of 3 blank plasmas that had undergone acetonitrile precipitation and evaporation with the results from DMB and DMV added to 3 fresh plasmas before deproteination and evaporation. Recoveries of QCL and QCH concentrations from plasma were 77.5% (CV 10.4%) and 62.1% (CV 6.1%), respectively.

3.2.5 Stability—The stability of a DMB stock solution kept at 4 °C in the dark for 6 months was 106.8% (CV 15.9%) (Table 3). The stabilities of DMB stock, as well as QCL, QCM, and QCH solutions in plasma at room temperature for 6 h are shown in Table 3. The stabilities of DMB in plasma during freeze-thaw cycling and for 5 months at −80 °C are also shown in Table 3. The stability of extracted samples kept in the autosampler for more than 37 h, ranged from 86.1 to 108.3% and did not change in any consistent manner with time.

3.3 Application of the assay to biological samples

The assay proved well suited to the quantitation of DMB in plasma of rats treated orally with a 500 mg/kg dose of DMB sodium salt (equivalent to 414 mg/kg of free DMB). As shown in

Fig. 3, maximum plasma DMB concentrations occurred at 2 h, but DMB concentrations were relatively constant at 400–600 µg/ml between 90 and 420 min after DMB administration. DMB concentrations in plasma decreased with a half-life of approximately 5 h (312 min), and DMB apparent clearance and volume of distribution was 1.45 ml/min/kg and 0.65 L/kg, respectively. These pharmacokinetic data confirm the longer half-life of DMB compared to butyrate. In addition, the volume of distribution of DMB is similar to the volume of distribution reported for butyrate (0.48–0.72 L/kg) [26], lending support to the validity of our data and assay, and providing additional impetus for clinical evaluation of DMB.

4. Conclusion

Our objective was to develop and validate an analytical method for the quantitation of DMB in plasma. This was accomplished by employing a procedure that derivatizes carboxylic acids to amides followed by LC with mass spectrometric detection. The method developed overcomes the challenges associated with the polar nature of DMB, its low molecular weight, and its lack of a chromophore or fluorophore. The method proved suitable for assessing the pharmacokinetics of DMB in rats and was an important adjunct to the toxicology studies upon which clinical trials of DMB are being based. The validation procedures described for rat plasma are being applied to human plasma so that the method can be used in the clinical development of DMB for the treatment of β -thalassemia and sickle cell disease.

Acknowledgements

Supported by NCI contract N01-CM52202, NHLBI and NIDDK under the NIH-RAID pilot program project (Susan P. Perrine, Project P.I.) and by NIH grant P30 CA47904. We also thank Mr. Jeremy A. Hedges for excellent secretarial assistance in preparation of this manuscript and the University of Pittsburgh Cancer Institute Hematology/Oncology Writing Group for constructive suggestions regarding the manuscript.

References

1. Redding-Lallinger R, Knoll C. *Curr Probl Pediatr Adolesc Health Care* 2006;36:346–376. [PubMed: 17055417]
2. Rund D, Rachmilewitz E. *N Engl J Med* 2005;353:1135–1146. [PubMed: 16162884]
3. Steinberg MH, Rodgers GP. *Medicine (Baltimore)* 2001;80:328–344. [PubMed: 11552087]
4. Cunningham MJ, Macklin EA, Neufeld EJ, Cohen AR. *Blood* 2004;104:34–39. [PubMed: 14988152]
5. Wonke B. *Semin Hematol* 2001;38:350–359. [PubMed: 11605170]
6. Perrine SP. *Hematology Am Soc Hematol Educ Program* 2005:38–44. [PubMed: 16304357]
7. Gallo E, Massaro P, Miniero R, David D, Tarella C. *Br J Haematol* 1979;41:211–221. [PubMed: 427031]
8. Perrine SP, Rudolph A, Faller DV, Roman C, Cohen RA, Chen SJ, Kan YW. *Proc Natl Acad Sci U S A* 1988;85:8540–8542. [PubMed: 2460870]
9. Liakopoulou E, Blau CA, Li Q, Josephson B, Wolf JA, Fournarakis B, Raisys V, Dover G, Papayannopoulou T, Stamatoyannopoulos G. *Blood* 1995;86:3227–3235. [PubMed: 7579419]
10. Liakopoulou E, Li Q, Stamatoyannopoulos G. *Blood Cells Mol Dis* 2002;29:48–56. [PubMed: 12482403]
11. Skarpidi E, Cao H, Heltweg B, White BF, Marhenke RL, Jung M, Stamatoyannopoulos G. *Exp Hematol* 2003;31:197–203. [PubMed: 12644016]
12. Constantoulakis P, Knitter G, Stamatoyannopoulos G. *Blood* 1989;74:1963–1971. [PubMed: 2478217]
13. Perrine SP, Miller BA, Faller DV, Cohen RA, Vichinsky EP, Hurst D, Lubin BH, Papayannopoulou T. *Blood* 1989;74:454–459. [PubMed: 2473801]
14. Boosalis MS, Bandyopadhyay R, Bresnick EH, Pace BS, Van DeMark K, Zhang B, Faller DV, Perrine SP. *Blood* 2001;97:3259–3267. [PubMed: 11342457]

15. Pace BS, White GL, Dover GJ, Boosalis MS, Faller DV, Perrine SP. Blood 2002;100:4640–4648. [PubMed: 12393583]
16. Castaneda S, Boosalis MS, Emery D, Thies A, Faller DV, Perrine SP. Blood Cells Mol Dis 2005;35:217–226. [PubMed: 16099182]
17. Pace BS, White GL, Dover GJ, Boosalis MS, Faller DV, Perrine SP. Blood 2002;100:4640–4648. [PubMed: 12393583]
18. Collins AF, Pearson HA, Giardina P, McDonagh KT, Brusilow SW, Dover GJ. Blood 1995;85:43–49. [PubMed: 7528572]
19. Ikuta T, Kan YW, Swerdlow PS, Faller DV, Perrine SP. Blood 1998;92:2924–2933. [PubMed: 9763579]
20. Dover GJ, Brusilow S, Charache S. Blood 1994;84:339–343. [PubMed: 7517215]
21. Atweh GF, Sutton M, Nassif I, Boosalis V, Dover GJ, Wallenstein S, Wright E, McMahon L, Stamatoyannopoulos G, Faller DV, Perrine SP. Blood 1999;93:1790–1797. [PubMed: 10068649]
22. Perrine SP, Castaneda SA, Boosalis MS, White GL, Jones BM, Bohacek R. Ann NY Acad Sci 2005;1054:257–265. [PubMed: 16339673]
23. Yeh KC, Kwan KC. J Pharmacokinet Biopharm 1978;6:79–98. [PubMed: 650423]
24. Rocci ML Jr, Jusko WJ. Comput Programs Biomed 1983;16:203–216. [PubMed: 6688572]
25. Kangani CO, Kelley DE. Tetrahedron Lett 2005;46:8917–8920. [PubMed: 16755305]
26. Egorin MJ, Yuan ZM, Sentz DL, Plaisance K, Eiseman JL. Cancer Chemother Pharmacol 1999;43:445–453. [PubMed: 10321503]

Fig. 1. Reaction of DMB and DMV with benzylamine to form N-benzyl-2,2-dimethylbutyramide and 2,2-dimethylpentanoic acid benzylamide.

Fig. 2.

Representative chromatograms monitoring: A) m/z 206 (DMB) and B) 220 (DMV internal standard). In figure 2A, the bottom chromatogram represents control rat plasma containing no DMB, the middle chromatogram represents control rat plasma containing 100 ng/ml DMB (16.3 min), and the top chromatogram represents control rat plasma containing 10,000 ng/ml DMB (16.3 min). In figure 2B, the bottom chromatogram represents control rat plasma containing no DMV internal standard, and the top chromatogram represents control rat plasma containing 500 ng/ml DMV internal standard (19 min).

Fig. 3. Concentrations of DMB in plasma of rats treated orally with 500 mg/kg of sodium DMB. Symbols represent the means $\pm$ SD of samples from 3 rats at each time point.

Table 1
Accuracy and precision of DMB calibration points from three successive triplicate standard curves in rat plasma.

	[DMB] (ng/ml)				
	100	300	1000	3000	10000
mean	101.0	294.3	1019.7	3113.7	9391.6
sd	5.40	12.47	45.68	167.28	700.87
CV ^a (%)	5.35	4.24	4.48	5.37	7.46

^a Coefficient of variation

Table 2

Assay performance data for the quantitation of LLQ, QCL, QCM and QCH DMB concentrations in rat plasma.

[DMB] (ng/ml)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)	Replicates
100	97.3	5.11	5.52	15
200	94.6	5.14	4.8	15
1000	97.6	3.37	5.57	15
7500	107.4	5.17	6.17	15

Table 3

Stability of DMB

Storage condition	Concentration (ng/ml)	Stability (%)	CV (%)	Replicates
Stock solution 6 months 4 °C	1000000	106.8	15.9	3
Plasma 3 freeze/thaw Cycles -80 °C	QCL	99.5	5.9	3
	QCM	106.9	11.6	3
	QCH	97.6	4.4	3
Plasma 5 months -80 °C	QCL	101.4	7.6	3
	QCM	103.1	5.8	3
	QCH	112.5	10.1	3
Plasma 6 h Ambient temperature	QCL	97.4	0.6	3
	QCM	106.8	6.7	3
	QCH	99.8	1.5	3