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Analysis of 2-(3-methyl-4-aminophenyl)-benzothiazole (NSC 674495) in plasma by gas chromatography with mass-selective detection

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Abstract

Certain naturally occurring isoflavonoids have been shown to inhibit protein-tyrosine kinases, and this has led to investigations of ring-modified structural analogs. Most recently, 2-(3-methyl-4-aminophenyl)-benzothiazole (MAB: NSC 674495) was shown to possess significant activity against certain breast cell cancer lines in vitro and in vivo. Our efforts thus focussed on developing a simple and sensitive method for quantitating MAB in plasma using GC–MS. The GC–MS assay was found to be linear over the range of 0.050 to 5.0 μg/ml, and was applied to monitor the plasma concentration of MAB in a rat dosed with 25 mg/kg as a 1 min intravenous infusion. Plasma was collected at intervals from 3 through 180 min, and concentrations of MAB were determined. Non-linear regression analysis of the plasma concentration-time data revealed that levels declined from a maximum at 3 min of 18 μg/ml to 1 μg/ml at 3 h in a biphasic manner. In another investigation, significant plasma concentrations of a major metabolite was detected and determined to be mono-*N*-acetylated MAB.

Keywords: Benzothiazole; 2-(3-Methyl-4-aminophenyl)-benzothiazole; NSC 674495

1. Introduction

When activated kinases were shown to be oncogene products, it was proposed that cell transformation was related to protein-tyrosine phosphorylation [1]. Considerable effort has subsequently been directed toward finding protein-tyrosine kinase inhibitors for evaluation as chemotherapeutic agents [2].

Early studies identified Genistein, a naturally occurring isoflavonoid, as possessing this inhibitory activity [3]. Subsequently, a progression of ring-modified structural analogs were investigated, including 2-phenylindoles [4,5], 2-phenylbenzo(b) furans [6], 2-phenylbenzo(b)thiophenes [7], and most recently, 2-phenylbenzothiazoles [8,9]. In particular,

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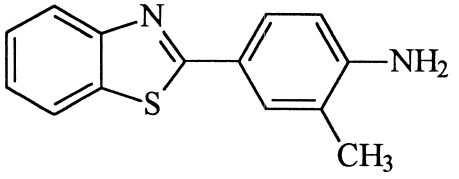

Fig. 1. Chemical structure of 2-(3-methyl-4-aminophenyl)-benzothiazole (MAB; NSC 674495).

2-(3-methyl-4-aminophenyl)-benzothiazole (MAB: NSC 674495 (Fig. 1)) was seen to have significant activity against certain breast cell cancer lines in vitro and in vivo [10]. Thus, our efforts focussed on characterizing the disposition of MAB and identifying its principal circulating metabolites in rat.

2. Experimental

2.1. Chemicals

2-(3-Methyl-4-aminophenyl)-benzothiazole (MAB: NSC 674495) was obtained from the Pharmaceutical Resources Branch, DTP, DCTDC, NCI (Bethesda, MD, USA). 2-(4-Dimethylamino)-phenyl-6-methylbenzothiazole (98%) (used as the internal standard) and acetic anhydride (99+%) were purchased from Aldrich (Milwaukee, WI, USA). All additional solvents (methanol, acetonitrile, acetone, pyridine, and *tert*.-butylmethyl ether) were all HPLC grade and were obtained from various sources. Human plasma was used for assay development and was acquired from the American Red Cross (Baltimore, MD, USA). Rat plasma was prepared by drawing blood from a Sprague-Dawley rat from the jugular vein via a pre-implanted catheter. The blood was first heparinized to prevent coagulation, and then centrifuged to pelletize the cells. The plasma was separated from the pellet and frozen at −78°C.

2.2. Synthesis of the metabolite, 2-(3-methyl-4-acetamidophenyl)-benzothiazole

To 10.0 mg (41.7 μmol) of MAB was added a mixture of acetic anhydride (0.5 ml) in pyridine (0.5 ml). The resulting mixture was briefly stirred and then left at ambient temperature overnight. Excess reagents were evaporated under a stream of nitrogen gas. Methanol (1.0 ml) was added [11] to the residue and the resulting mixture was sonicated for 40 min. The methanol was evaporated under a stream of nitrogen gas and the resulting residue was dried overnight under vacuum (20 mmHg) at ambient temperature to give 11.7 mg of crystalline 2-(3-methyl-4-acetamidophenyl)-benzothiazole (41.4 μmol, 99%), mp 207.1–207.3°C. ^{1}H-NMR (DMSO-d_6): δ 9.40 (s,1H (exchangeable)), 8.11 (d,1H), 8.02 (d,1H), 7.94 (s,1H), 7.88 (d,1H), 7.77 (m,1H), 7.52 (t,1H), 7.44 (t,1H), 2.34 (s,3H), 2.10 (s,3H). COSY experiments demonstrated strong correlations among the group of signals observed at δ 8.11, 8.02, 7.52 and 7.44, and among the signals observed at δ 7.88 and 7.77. Mass spectrum (electron-ionization, 70 eV) *m*/*z* (% relative abundance): 282 ($M^{+\cdot}$,55), 240 (100), 223 (3), 109 (6), 108 (4), 69 (9). HRMS: *m*/*z* 282.0826 (calculated for $C_{16}H_{14}N_2OS$: 282.0827). **Analysis.** Calculated for $C_{16}H_{14}N_2OS$: 0.25; CH_3OH: C, 67.22; H, 5.21; N, 9.65. Found: C, 67.34; H, 5.31; N, 9.67.

2.3. Sample preparation

A 100 μl aliquot of human plasma and 3 ml of *tert*.-butyl methyl ether solution containing 0.002 μg of 2-(4-dimethylaminophenyl)-6-methylbenzothiazole (the internal standard (I.S.)) per ml of *tert*.-butyl methyl ether (TBME) were pipetted into a 10 ml centrifuge tube and tightly capped. After vigorous mixing on a vortex action stirrer for 1 min, the sample was extracted for an additional 10 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, USA), and then centrifuged for 4 min at 2500 *g*. The upper organic layer was carefully transferred to a round-bottomed culture tube and concentrated to dryness under a stream of nitrogen gas using a Meyer N-EVAP (Organomation Assoc., Berlin, MA, USA), with a bath temperature of 45–50°C. The residue was reconstituted with 100 μl of acetone, then transferred into a borosilicate glass conical insert which was placed in a 12×32 mm autosampler vial and sealed with a silicone/PFTE Snap-Cap (Scientific Resources, Somerset, NJ, USA). A 5 μl aliquot of the reconstituted extract was injected into the chromatograph.

2.4. Sample analysis

Analyses were performed using a 5890 Series II gas chromatograph equipped with a capillary inlet and a 5971A Mass Selective Detector, controlled through a DOS-series MS ChemStation (Hewlett-Packard, Palo Alto, CA, USA). The inlet was fitted with a deactivated quartz direct injection liner (2.0 mm, I.D., 140 μl volume) and operated in the splitless mode. Samples (5 μl) were introduced into the gas chromatograph using a Model 7673 automatic injector (Hewlett-Packard) with a 10 μl syringe (Hamilton, Reno, NV, USA). Acetonitrile was used as the injector syringe wash solvent. Separations were performed on a 15 m×0.25 mm fused-silica capillary column wall-coated with 0.25 μm DB-1 cross-linked dimethylpolysiloxane (J&W Scientific, Folsom, CA, USA). Helium was employed as the carrier gas at a linear velocity of 27.5 cm/s (pentane, 60°C) using flow-rates of 3 and 35 ml/min at the septum purge and split vents, respectively. Temperatures were 285°C at the injection port and 290°C at the transfer line to the detector. Injections were made at an initial oven temperature of 150°C. The inlet purge was activated at 1.0 min postinjection. The oven temperature was held isothermally at 150°C for 2 min, then increased linearly to 260°C at 10°C/min and finally ramped from 260°C to 280°C at 20°C/min. The final temperature was maintained for 5 min to desorb less volatile components of the sample. Mass spectral detection (electron-ionization, 70 eV) was performed by selected ion monitoring, sequentially measuring ions at m/z 240 and 268, with a 75 ms dwell time. Data was collected between 5 and 19 min postinjection. The ion chromatograms were integrated to provide peak areas.

2.5. Quantitation

A series of seven rat plasma standards which composed the standard curve (0.0503, 0.126, 0.252, 0.503, 1.257, 2.52, and 5.03 μg/ml) were prepared for analysis and run together with pharmacokinetic plasma specimens on a daily basis. Ratios of the chromatographic peak area for MAB to that of the I.S. were calculated. Standard curves were constructed by plotting the peak area ratio of the analyte to the internal standard against the concentration of the analyte.

Linear least squares regression was performed using a weighting factor of $1/y_{obs}$, without inclusion of the origin, to determine the slope, y-intercept and correlation coefficient of the best fit line. Analyte concentrations in unknown samples were calculated using results of the regression analysis. Each unknown sample was initially assayed in duplicate on separate days, with additional analyses performed if the replicate determinations deviated from their average by more than 10%. Specimens with concentrations exceeding the upper limit of the standard curve were reassayed upon appropriate dilution with blank plasma.

2.6. Assay validation

Accuracy and repeatability of the assay were assessed by analyzing the backcalculated sample concentrations and regression parameters from a series of calibration curves of MAB in plasma that were prepared and analyzed on separate days. The relative standard deviation (RSD) of the mean predicted concentrations for the independently assayed standards provided the measure of repeatability. The lower limit of quantitation was defined as the minimum concentration amenable to analysis with an inter-day RSD not exceeding 20%. Accuracy of the assay was assessed by expressing the mean predicted analyte concentration as a percentage of its known concentration in the standard solutions.

3. Results and discussion

3.1. Specificity of method

As MAB emerged from a capillary gas chromatographic column, its EI (70 eV) mass spectrum (Fig. 2A) was acquired and then examined. The most striking feature of the mass spectrum of MAB is that a significant portion of the ion current resides in the molecular ion (m/z 240), which is the base peak (100%) of the spectrum. A few product ions, whose origins may be rationalized in terms of complex fragmentation of MAB's aromatic rings or ring substituents, are also present, but none possess a

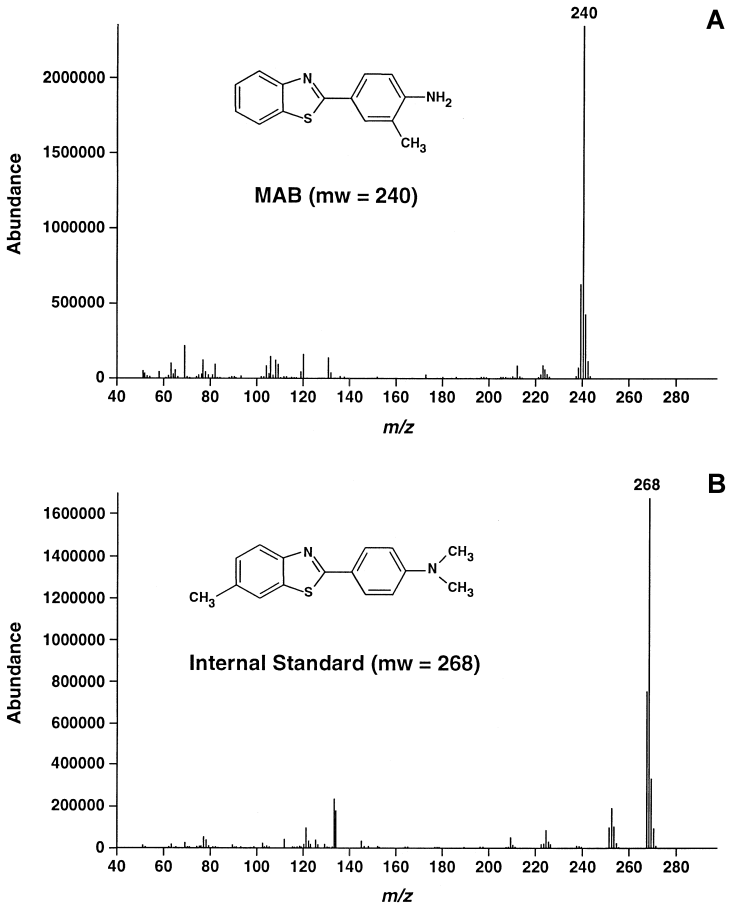

Fig. 2. Electron ionization (70 eV) mass spectra of (A) MAB and (B) 2-(4-aminophenyl)-6-methylbenzothiazole, the internal standard (I.S.) used in the assay.

relative abundance greater than 15%. The mass spectrum of the internal standard (Fig. 2B) displayed features similar to that of the mass spectrum of MAB. Thus, selected ion detection at m/z 240 was used in developing a highly specific and sufficiently sensitive method for quantitating MAB in plasma.

Capillary gas chromatographic conditions were determined in order to facilitate the analysis of MAB, which was isolated from human plasma by extraction into *tert.*-butyl methyl ether. Shown in Fig. 3 are time-selected ion profiles reconstructed from data acquired during gas chromatographic separation with selected ion detection of (A) an extract of human plasma spiked with MAB at a concentration of 0.503 μg/ml and (B) an extract of human plasma to which only the I.S. had been added. No peaks of endogenous origin that would potentially interfere with detection of MAB were observed in chromatograms of either human or rat plasma extracts.

3.2. Linearity of calibration and inter-day reproducibility

The MAB-to-I.S. chromatographic peak area ratio was found to be directly proportional to the added concentration of MAB in human plasma from about 0.05 to 5.0 μg/ml (0.21 to 21.0 μ*M*). Mean values (±SD) of the linear regression parameters for 12 calibration curves of MAB in human plasma, independently prepared and assayed over an 8-week period were: slope, 14.48±1.33 ml/μg; *y*-intercept,

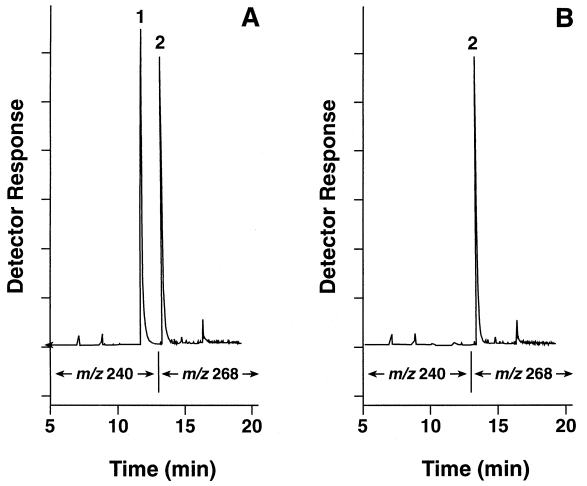

Fig. 3. Time-selected ion profiles reconstructed from data acquired during gas chromatographic separation with selected ion detection of (A) an extract of human plasma spiked with MAB at a concentration of 0.503 μg/ml and (B) an extract of human plasma to which only the I.S. had been added. Peak assignments: 1, MAB; 2, I.S.

-0.42 ± 0.17; correlation coefficient, 0.988 ± 0.005. Coefficients of variation of the mean predicted MAB concentrations ranged from 3.1 to 12.2%.

3.3. Accuracy and repeatability

Backcalculated sample concentrations were analyzed from 12 different calibration curves of MAB in human plasma independently prepared and analyzed over an 8-week period. Accuracy of the assay was assessed by expressing the mean predicted analyte concentration as a percentage of its known concentration in the standard solutions, whereas repeatability reflects inter-day variation. As shown in Table 1, the RSD for inter-day quantitation of MAB in human plasma was ≤13% for all concentrations included in the standard curve. The mean recovery of MAB in human plasma standards ranged from about 85 to 107% of the added concentration for concentrations ≥0.126 μg/ml.

3.4. Analyte stability

A human plasma standard of MAB (2.52 μg/ml) was incubated for 2 days at 37°C. At selected times, an aliquot of the plasma mixture was removed and analyzed for remaining MAB. After 2 days' incubation at 37°C, the concentration of MAB had declined to 2.25 μg/ml, indicating that about 90% of the MAB remained. In a separate experiment, another sample (2.52 μg/ml human plasma standard of MAB) was stored at −70°C and was, at selected times, similarly sampled and analyzed for remaining MAB. No significant change in the concentration of MAB in the human plasma sample was noted after 1 month of storage at −70°C.

Table 1
Accuracy and repeatability for assaying MAB in human plasma[a]

Concentration added (μg/ml)	MAB	
	Accuracy (%)	Repeatability (%)
5.03	107.0	3.09
2.52	100.5	5.69
1.257	92.1	4.93
0.503	84.8	10.25
0.252	86.1	5.74
0.126	93.1	5.17
0.0503	144.8	12.16

[a] Accuracy and repeatability of the assay were assessed from 12 standard curves of MAB in human plasma analyzed over an 8-week period.

3.5. Lower limit of quantitation

The lowest point of the matrix calibration curve which is both repeatable (5.2%, $n=12$) and accurate (93.1%) is the 0.126 μg/ml human plasma sample. MAB is detectable at significantly lesser quantities: injection of 50 pg of MAB (corresponding to a processed 0.016 μg/ml human plasma sample) into the GC–MS produces a signal which is easily integrated (signal-to-noise ratio=20). Analysis of the 0.050 μg/ml human plasma sample is seen to be highly repeatable (12.2%), but is no longer accurate (145%). Thus, the lower limit of detection (LOD) of MAB is 0.016 μg/ml, and the lower limit of quantitation (LOQ) in human plasma is 0.126 μg/ml.

3.6. Absolute recovery

Five pairs of calibration curves were independently prepared and analyzed on separate days. Each pair

of calibration curves consisted of a set of seven standard samples of MAB in matrix (human plasma) and in non-matrix. Comparing absolute detector responses for the I.S. in matrix and non-matrix shows an extraction efficiency of 98.8% for the I.S. For MAB, the matrix calibration curves gave an average slope of 15.75±2.25, and the non-matrix calibration curves gave an average slope of 24.66±2.51. The ratio of the slopes therefore provides the measure of absolute recovery (63.9%) for MAB. Recovery is incomplete, suggesting perhaps the binding of MAB to matrix components, such as proteins. However, a mean value for the relative recovery of MAB, calculated across the concentration range of 0.126 to 5.03 μg/ml was 93.9±7.8%, which indicates consistent extraction from human plasma.

3.7. Preliminary plasma pharmacokinetics

A rat was given 25 mg/kg MAB as a 1 min intravenous infusion. Plasma was collected at intervals from 3 through 180 min, and concentrations of the compound were determined. Nonlinear regression analysis of the plasma concentration-time data (Fig. 4) revealed that levels declined in a biphasic manner, from a maximum at 3 min of 18 μg/ml (75 μ*M*) to 1 μg/ml (4 μ*M*) at 3 h. The half-lives for the initial and terminal phases were 3.3 and 87.5 min, respectively.

3.8. Identification of MAB and metabolites

Plasma samples acquired from a rat following intravenous administration of 25 mg/kg MAB were analyzed by GC–MS (Fig. 5) using mass scanning detection. Components such as lipids which are normally present in plasma were identified by their mass spectra. By inspecting the total ion chromatogram, the presence of MAB and its principal circulating metabolite (subsequently determined to be 2-(3-methyl-4-acetamidophenyl)-benzothiazole) became obvious (Fig. 5A). Authentic MAB was then *N*-acetylated and analyzed by GC–MS (Fig. 5B), and the chromatographic retention time and mass spectrum of the synthesized material corresponded to that of the circulating rat metabolite.

4. Conclusions

A specific assay for determining MAB in human or rat plasma has been developed. The method involves preliminary isolation of the compound from

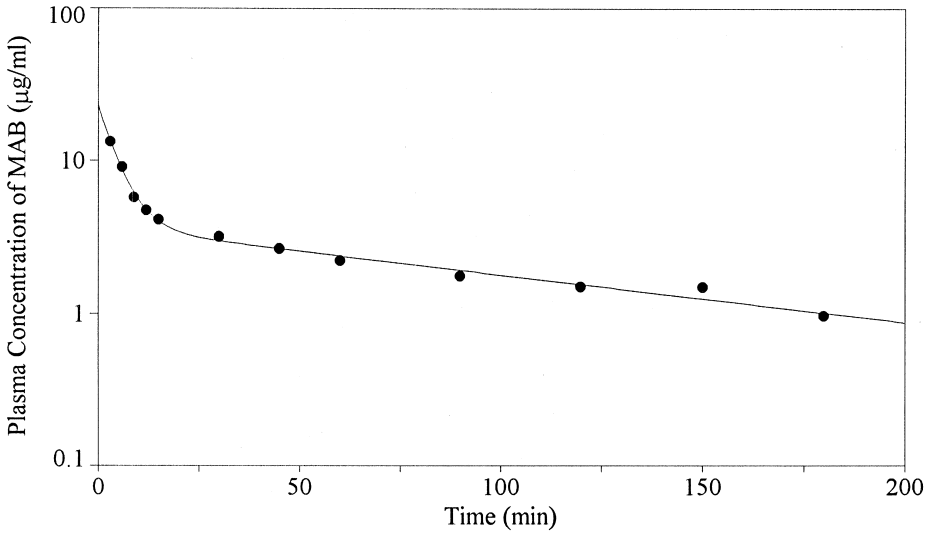

Fig. 4. Plasma concentration-time profile of MAB determined following intravenous injection to a rat (25 mg/kg). The GC–MS method was used for the analysis of serial plasma specimens acquired from the rat.

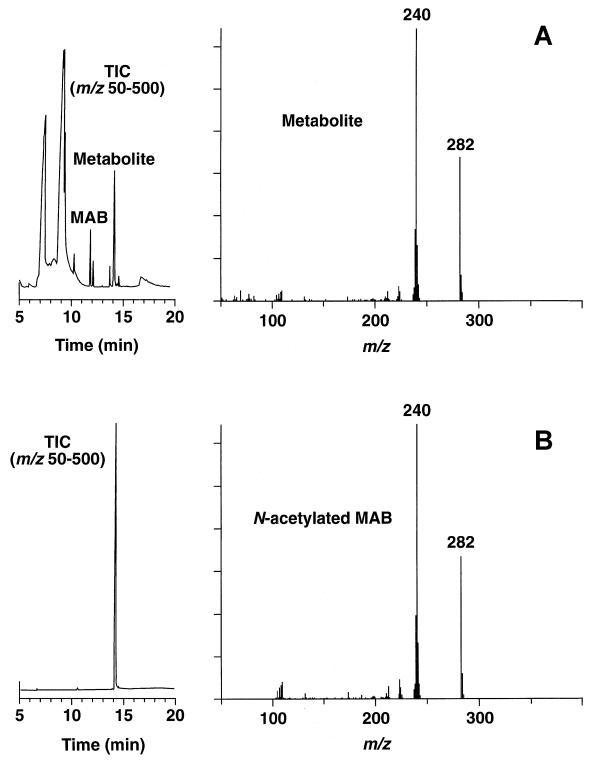

Fig. 5. Selected plasma specimens acquired following intravenous injection of MAB to a rat were pooled, extracted, and examined by GC–MS with scanning detection. MAB was easily discerned from endogenous materials, as well as an apparent metabolite (A), whose chemical structure was proposed to be that of *N*-acetylated MAB. GC–MS analysis of a sample of MAB which had been subjected to *N*-acetylation (B) gave a peak which possessed the same retention time and mass spectrum as that of the metabolite.

plasma by extraction into TBME. Following separation on GC and detection by EI–MS with selected ion monitoring, the lowest concentration of MAB that could be quantified with acceptable reproducibility (RSD<20%) in 100 μl of plasma was near 0.0503 μg/ml. The assay has been shown to be specific, accurate and reproducible, thereby rendering the procedure appropriate for monitoring plasma levels of the drug during pharmacokinetic studies.

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[11] Acetylating a primary amine using a mixture of acetic anhydride and pyridine frequently results in a mixture of mono-*N*- and di-*N,N*-acetylated products. The latter is a simple imide, which is not very stable in the presence of weak nucleophiles such as methanol, whereas the former is an amide, and is quite stable under these conditions. Treating the imide with methanol generates the mono-*N*-acetylated product as well as methyl acetate, which is highly volatile and easily removed from the reaction mixture. Thus, acetylating a primary amine using a mixture of acetic anhydride and pyridine, followed by treating the product mixture with methanol leads to a nearly quantitative yield of a highly pure mono-*N*-acetylated product.