ELSEVIER

Journal of Chromatography B, 718 (1998) 193–198

JOURNAL OF CHROMATOGRAPHY B

Short communication

Determination of phenethylamine, a phenethyl isothiocyanate marker, in dog plasma using solid-phase extraction and gas chromatography-mass spectrometry with chemical ionization

Adam Negrusz[1,a,*], Christine M. Moore[b], Nancy S. McDonagh[a], Eugene F. Woods[a], James A. Crowell[c], Barry S. Levine[d]

[a]*Department of Pharmaceutics and Pharmacodynamics (M/C 865), College of Pharmacy, The University of Illinois at Chicago, 833 S. Wood St., Chicago, IL 60612, USA*

[b]*United States Drug Testing Laboratories, Inc., 1700 S. Mount Prospect Rd., Des Plaines, IL 60018, USA*

[c]*Division of Cancer Prevention & Control, National Cancer Institute, Rockville, MD 20892, USA*

[d]*Toxicology Research Laboratory, Department of Pharmacology, College of Medicine, The University of Illinois at Chicago, 1940 W. Taylor St., Chicago, IL 60612, USA*

Received 24 March 1998; received in revised form 10 July 1998; accepted 17 July 1998

Abstract

Phenethyl isothiocyanate is unstable in aqueous media and at low pH, and rapidly degrades to phenethylamine. Concentrations of phenethylamine, a phenethyl isothiocyanate marker, in dog plasma, were determined utilizing solid-phase extraction and gas chromatography–mass spectrometry with chemical ionization using acetone as the reagent gas. Deuterated d_5-amphetamine was used as an internal standard. After extraction, phenethylamine and d_5-amphetamine were derivatized using MBHFBA. Ions monitored for d_5-amphetamine were m/z 337 and 338; and for phenethylamine were m/z 318 and 319. Precision and accuracy were studied using control solutions prepared in naive dog plasma (80 and 300 ng/ml). Intra-day variability was determined using six replicates of each control solution analyzed on a single day. The relative standard deviation for the 80 ng/ml control was 12.9% and for the 300 ng/ml it was 12.1%. Relative accuracy was 10.9% for the low control and −4.1% for the high control. Inter-day variability was determined over a 6-day period. For the 80 and 300 ng/ml control solutions, the relative standard deviations were 15.8 and 9.1%, respectively, and relative accuracy values were 10.1 and −5.2%, respectively. Standard curves were prepared in naive dog plasma and were linear over the range of phenethylamine assayed (10–500 ng/ml). The results of this study indicate that the proposed method is simple, precise, accurate and sensitive enough for analysis of large numbers of plasma samples.

Keywords: Phenethylamine; Phenethyl isothiocyanate

*Corresponding author.

[1]Analytical work was performed in the UIC Department of Pharmaceutics and Pharmacodynamics, and in the United States Drug Testing Laboratories, Inc., 1700 S. Mount Prospect Rd., Des Plaines, IL 60018, USA.

0378-4347/98/$19.00
PII: S0378-4347(98)00354-5

1. Introduction

The possibility of using naturally occurring anticarcinogens as cancer chemopreventives has recently generated a great deal of research. Of particular interest to phytochemical researchers are foods that prevent or arrest the development of cancer.

Phenethyl isothiocyanate (PEITC) is found in cruciferous vegetables such as cabbage, turnips, radishes, and watercress [1]. These foods release sulforaphanes and related isothiocyanates from their glucosinolate precursors when consumed, and are thought to prevent tumorigenesis by inducing the production of phase II detoxifying enzymes, for example, glutathione transferases and epoxide hydrolase [2], and by inhibition of phase I enzymes, respectively.

In addition to screening tests to evaluate antitumor activity, studies have been conducted with laboratory animals which confirm the action of PEITC on the prevention of tumors [1,3,4]. Pretreatment with PEITC inhibited induction of mammary tumors in rats by the carcinogen 7,12-dimethylbenz[*a*]anthracene, and dietary PEITC inhibited forestomach and pulmonary adenomas similarly induced in mice [1]. Another study tested PEITC for effectiveness of inhibition of tumorigenicity and DNA methylation induced by the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-3(pyridyl)-1-butanone (NNK), in the lungs of mice [4]. Mice that received PEITC via gavage showed inhibition of both tumor development and DNA methylation in lungs. Investigation into the mechanism of tumor inhibition in rats, where tumors were induced by NNK, indicated that PEITC does not alter the amount of NNK reaching the lung. This finding supports the hypothesis that inhibition of NNK-induced tumorigenesis by PEITC is a result of decreased metabolic activation of NNK [5]. It is believed that PEITC inhibits the microsomal metabolism of nitrosamines [4].

In aqueous media, and at low pH, PEITC spontaneously degrades to phenethylamine (PEA). Experiments by others to analyze PEITC with HPLC have been unsuccessful due to the rapid degradation of the drug [6].

PEA, the simplest trace amine metabolite of phenylalanine, is thought to act as a neuromodulator in the central nervous system of mammals, where it modulates dopaminergic and noradrenergic transmission and affects the release and uptake of dopamine and noradrenaline [7]. It has been suggested that a functional deficiency of PEA may be involved in the etiology of affective disorders, and PEA may have a relationship with depression and the action of antidepressants [8].

PEA has been considered to be the basic carbon skeleton of sympathomimetic amines since the early 1900s. It has a pharmacological profile similar to that of amphetamine, and is the parent compound for abused drugs in the amphetamine class.

A wide variety of foods contain PEA, but the diet is probably not a significant contributor to endogenous levels. Experiments with rats [9] and cats [10] have shown the intestines to be capable of substantial pre-systemic elimination of PEA.

PEA is degraded by platelet monoamine oxidase type B (MAO-B), for which PEA is a preferred substrate. The first step in its metabolism is the oxidative deamination by MAO-B to phenylacetaldehyde. The phenylacetaldehyde is oxidized to phenylacetic acid, which is then followed by species-dependent conjugation with glutamine or glycine [9]. Endogenous levels of PEA in mammals are very low [11].

The goal of this project was to develop a means of analysis for the determination of PEA, a PEITC marker, in dog plasma that would be sensitive, specific, and suitable for analyzing large numbers of plasma samples during toxicity studies of PEITC in dogs. Gas chromatography–mass spectrometry was used and chemical ionization was chosen for its soft ionization, which has the bonus of high sensitivity due to the less efficient detection of biological matrix interferences compared to the analyte. The presented study was performed in compliance with Good Laboratory Practice regulations.

2. Experimental

2.1. Materials

Phenethylamine hydrochloride, butyronitrile (99%), and sulfuric acid (ACS grade) were purchased from Aldrich Chemical Company (Mil-

waukee, WI, USA); methanol (purified grade), acetonitrile (ACS grade), acetic acid (USP grade), methylene chloride (ACS grade), ammonium hydroxide (reagent grade), and isopropyl alcohol (ACS grade) were purchased from Columbus Chemical Industries (Columbus, OH, USA); d_5-amphetamine, concentration 100 μg/ml in methanol, was obtained from Radian Corporation (Austin, TX, USA); potassium phosphate dibasic (ACS grade), potassium phosphate monobasic (ACS grade) and acetone (HPLC grade) were purchased from Fisher Scientific (Itasca, IL, USA). Isolute™ HCX extraction columns (200 mg of sorbent) were obtained from Jones Chromatography (Lakewood, CO, USA). MBHFBA (*N*-methyl-bis-heptafluorobutyric amide) was purchased from Macherey–Nagel (Dwer, Germany). Blank beagle dog plasma was supplied by the Toxicology Research Laboratory, Department of Pharmacology, College of Medicine, University of Illinois at Chicago (Chicago, IL, USA). Saline USP (0.9% NaCl) was purchased from Baxter (McGraw, IL, USA). A one liter volume of phosphate buffer, pH 6, was prepared by transferring 11.9 g of potassium phosphate monobasic and 2.1 g of potassium phosphate dibasic into a one liter volumetric flask, dissolving in approximately 500 ml of deionized water and diluting to mark with deionized water.

2.2. Instrumentation and conditions

The GC–MS system used was a Varian Star 3400 Saturn 2 (Walnut Creek, CA, USA) equipped with the ion trap working in chemical ionization mode and a Varian 8200 Autosampler. Acetone was used as the reagent gas. The analytical capillary column (DB-5MS, 25 m, 0.25 mm I.D., 0.25 μm film thickness) was purchased from J&W Scientific (Folsom, CA, USA). The carrier gas was helium (ultra high purity 99.999%), and the flow-rate was 1 ml/min. The sample volume used was 3 μl; injecton time, 0.1 min; injection rate, 10 μl/s; needle depth, 95%; syringe wash time, 10 s; uptake speed, 2 μl/s. The injector temperature was set at 150°C and the oven was programmed as follows: 100°C for 1 min; 100–190°C, rate 20°C/min; 190°C for 4.5 min. The transfer line temperature was kept at 300°C and the instrument was operated in full scan mode. For the detector, the following conditions were applied: multiplier set voltage at 2500 V; manifold temperature, 250°C; low mass, 270 *m/z*; high mass, 399 *m/z*; scan rate, 460 ms; segment acquire time, 5.5 min; peak threshold, 5 counts; filament/multiplier delay, 3.5 min; mass defect, 100 mu/100 u; background mass, 268 *m/z*. In this experiment, *m/z* 337 and 338 ions were monitored for d_5-amphetamine (internal standard) and *m/z* 318 and 319 were monitored for phenethylamine.

2.3. Analytical method

A five-point standard curve for phenethylamine was prepared in naive dog plasma and concentrations were as follows: 10, 25, 100, 250, and 500 ng/ml. In addition, the following two levels of control solutions of phenethylamine in dog plasma were prepared: 80 and 300 ng/ml. Aliquots (1 ml) of standard and control samples were transferred to individually labelled plastic centrifuge tubes. Methanol (250 μl), as well as the internal standard, d_5-amphetamine solution (50 μl, 2 μg/ml), were added to each sample. Next, an aliquot (1 ml) of acetonitrile was added to each control and standard solution. All tubes were then vortex mixed for 5–10 s and allowed to stand at room temperature for 15 min. All specimens were centrifuged for 5 min at 5000 *g* and inspected for evidence of a protein emulsion after centrifugation. If an emulsion was observed, the supernatant was gently stirred with a wooden applicator stick and re-centrifuged. The clear supernatant was poured into a plastic test tube and phosphate buffer (9 ml, 0.1 *M*, pH 6) was added. Solid-phase HCX extraction columns were conditioned by adding the following sequentially: methanol (3 ml), deionized water (3 ml), and phosphate buffer (1 ml of 0.1 *M*, pH 6). Phenethylamine extraction was performed as follows: (1) the supernatant was added to the conditioned column and drawn through under minimum vacuum; the column was washed with deionized water (2 ml) and then acetic acid (1 ml, 1 *M*) was drawn through slowly with minimum vacuum; (2) the column was dried for 5 min at maximum vacuum; (3) the column was washed with methanol (3 ml) drawn through at a moderate rate; (4) the column was dried for 2 min under maximum vacuum; (5) phenethylamine and the internal standard

were eluted under gravity only by application of the elution solution (3 ml), isopropanol–methylene chloride–concentrated ammonium hydroxide (78:20:2, v/v/v).

The extracts were evaporated to dryness at 60°C in the tubes into which they were collected. Two drops of sulfuric acid (1%, v/v) were added to each tube 2 min after evaporation was begun. The dried samples were reconstituted in the same tubes using butyronitrile (50 μl), then transferred to autosampler vials. The vials were capped, and derivatizing agent, MBHFBA (10 μl) was added to each with a gas-tight syringe. The vials were then heated at 80°C for 20 min, cooled down and placed on the autosampler for GC–MS analysis.

3. Results

Quantitation of PEA was performed using an internal standard method. The standard curve was determined by linear least-squares regression analysis of the ratio of peak area of PEA to the peak area of the internal standard, d_5-amphetamine, as a function of concentration. Peak area ratios were determined for controls and used to calculate the concentrations of PEA. Varian Saturn version 5.0 computer software was used for calculations. The standard curve for phenethylamine was linear over the range of 10–500 ng/ml, had a regression coefficient of 0.995, a slope of 7.813×10^{-3}, a S.D. of slope of 4.260×10^{-4}, an intercept of 0.259, a S.D. of intercept of 0.108, a standard error of 0.174, and five data points. The limit of detection (LOD) for the GC–MS instrumentation was 5 ng/ml of PEA in dog plasma, and it was the lowest phenethylamine concentration at which 'goodness of fit' criteria were met and signal-to-noise ratio was greater than 5. The limit of quantitation (LOQ) was arbitrarily established to be 10 ng/ml, the lowest phenethylamine concentration on the standard curve. Precision and accuracy studies were conducted using two concentrations of control solutions of phenethylamine prepared in naive dog plasma, low control (80 ng/ml) and high control (300 ng/ml). Intra-day variability was determined using six replicates of each control solution analyzed on a single day, and inter-day variability was determined over a 6-day period

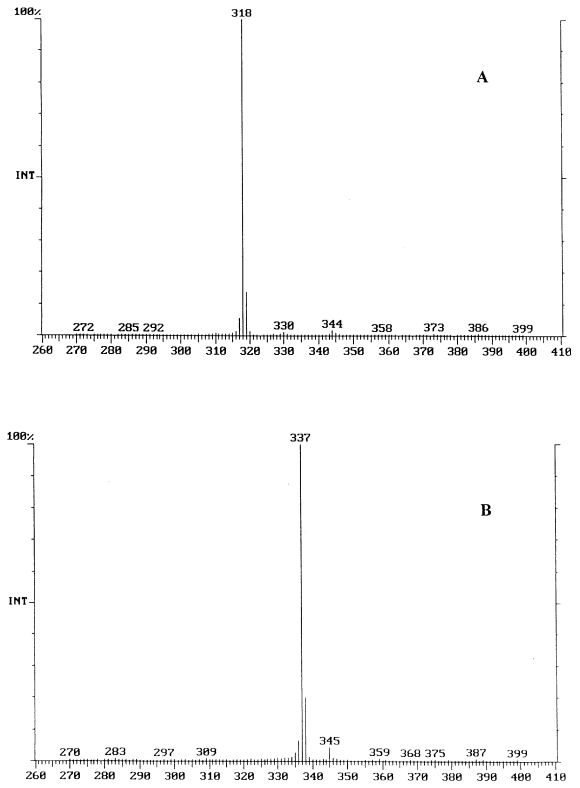

Fig. 1. Chemical ionization mass spectrum of phenethylamine (A), and d_5-amphetamine (B), extracted from dog plasma.

Table 1
Accuracy and precision of phenethylamine control concentrations prepared in blank dog plasma (ng/ml)

Parameter	Intra-day ($n=6$)		Inter-day ($n=15$)	
	Low control[a]	High control[b]	Low control[a]	High control[b]
Mean measured concentration (±S.D.)	88.7 (±11.5)	287.6 (±34.7)	88.1 (±13.9)	284.4 (±25.8)
% R.S.D.	12.9	12.1	15.8	9.1
% Relative accuracy	10.9	−4.1	10.1	−5.2

[a]Theoretical concentration, 80.
[b]Theoretical concentration, 300.

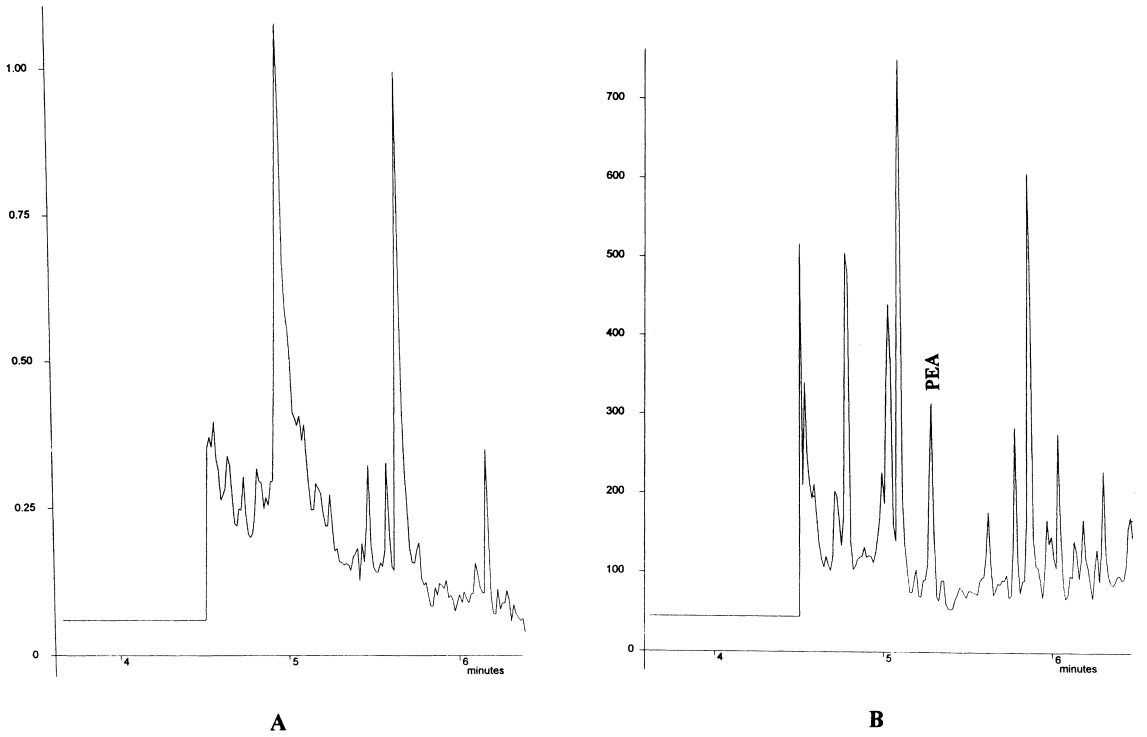

Fig. 2. Total ion chromatogram (full scan mode) of a blank dog plasma extract (A), and the extract of the dog plasma spiked with phenethylamine (B), at a concentration of 80 ng/ml.

by analyzing replicates of each control solution. Table 1 summarizes results of intra- and inter-day precision and accuracy studies. Relative accuracies (%) were calculated using the following equation:

(Mean measured concentration − Theoretical concentration)/(Theoretical concentration) × 100%.

All analytical parameters presented in Table 1 are acceptable and do not show significant variability of results obtained. Fig. 1A and Fig. 1B present full scan chemical ionization mass spectra of PEA and d_5-amphetamine, respectively, extracted from dog plasma. Fig. 2A and Fig. 2B show total ion chromatogram of a blank extract and a spiked extract, respectively, recorded in a full scan mode.

4. Discussion

The experimental method, solid-phase extraction with HCX columns and analysis by ion trap GC–MS with chemical ionization operating in a full scan mode, proved to be satisfactory for PEA quantification. The standard curve was shown to be linear over the range of 10–500 ng/ml, while the instrumentation was capable of detecting concentrations of at least as low as 5 ng/ml. Precision and accuracy results (see Table 1) were acceptable and, in fact, compare favorably with precision and accuracy results generated by other methods of analysis of PEA concentration in plasma. A method in which PEA concentrations were determined in human plasma and rat brain, utilizing solvent extraction with cyclohexane and HPLC with amperometric electrochemical detection, resulted in relative standard deviations of 16.1% intra-day and 40.6% inter-day [12]. In another experiment, dog plasma PEA levels were determined using solvent extraction (benzene) with gas chromatography and flame ionization detection [11,13]. The assay was linear throughout the range tested, 0.1–50 μg/ml, and had a low detection limit of 0.05 μg/ml for PEA. PEA was also extracted from rat liver, brain and blood with di-(2-ethylhexylphosphate) (2.5%, w/v) in chloroform and

gas chromatography with electron capture detection was employed [8]. Durden and Davis presented an analytical method for PEA in rat brain and human and dog plasma using solvent extraction (ethyl acetate–hexane) and ultra-sensitive negative chemical ion GC–MS [14]. The solid-phase extraction with GC–MS presented in this paper yielded superior results. Liquid–liquid extraction of PEA from biological fluids appears to be the most widely used method. Solvents such as chloroform, benzene, or hexane are commonly used. The solid-phase extraction method is clearly more friendly to the analyst and to the environment.

GC–MS analysis is another advantage to this procedure. It is a more sensitive and specific technique than others mentioned in the literature for PEA analysis, such as HPLC with UV detection or gas chromatography with flame ionization detection. In addition, the use of solid-phase extraction provides virtually 100% recovery of both PEA and the internal standard.

In summary, the proposed method of quantitation of PEA in dog plasma was demonstrated to be simple, precise, accurate and sensitive enough for analysis of large numbers of plasma samples.

Acknowledgements

This work was supported by Contract No. N01-CN-25508-01 from the Chemoprevention Branch of the National Cancer Institute.

References

[1] K. Eklind, M. Morse, F. Chung, Carcinogenesis 11 (1990) 2033.
[2] Y. Zhang, T. Kensler, C. Cho, G. Posner, P. Talalay, Proc. Natl. Acad. Sci. USA 91 (1994) 3147.
[3] M. Staretz, S. Hecht, Cancer Res. 55 (1995) 5580.
[4] M. Morse, K. Eklind, S. Hecht, K. Jordan, C. Choi, D. Desai, S. Amin, F. Chung, Cancer Res. 51 (1991) 1846.
[5] M. Staretz, S. Hecht, Cancer Res. 55 (1995) 5580.
[6] W. Mullin, J. Chromatogr. 155 (1978) 198.
[7] L. Kruse, C. Kaiser, W. DeWolf, J. Frazee, S. Ross, J. Wawro, M. Wise, K. Flaim, J. Sawyer, R. Erickson, M. Ezekiel, E. Ohostein, B. Berkowitz, J. Med. Chem. 30 (1987) 486.
[8] G. Baker, R. Coutts, T. Rao, Br. J. Pharmacol. 92 (1987) 243.
[9] P.J. Worland, K.F. Ilett, J. Pharm. Pharmacol. 35 (1983) 636.
[10] G. Garcha, P.R. Imrie, E. Marley, D.V. Thomas, Br. J. Pharmacol. 86 (1985) 877.
[11] E. Cone, M.E. Risner, G.L. Neidert, Res. Commun. Chem. Pathol. Pharmacol. 22 (1978) 211.
[12] N.D. Huebert, V. Schwach, G. Richter, M. Zreika, C. Hinze, K. Haegele, Anal. Biochem. 221 (1994) 42.
[13] H.E. Shannon, E.J. Cone, D. Yousefnejad, J. Pharmacol. Exp. Ther. 223 (1982) 190.
[14] D.A. Durden, B.A. Davis, Neurochem. Res. 9 (1993) 995.