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Determination of Gamma Aminobutyric Acid (GABA) in Horse Plasma

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ABSTRACT

Gamma aminobutyric acid (GABA) is a main inhibitory neurotransmitter in adult mammalian nervous systems (1, 2). It is closely associated with glutamate, a main excitatory neurotransmitter in a complex relationship called the glutamine-GABA-glutamate cycle (3–5). Their activity is independent of the catecholamine neurotransmitters, dopamine and noradrenaline. It is now known that imbalances in the glutamine-GABA-glutamate cycle are associated with a variety of disease states such as epilepsy, autism, and alcoholism, and drugs used to treat these conditions elevate GABA in the CNS (6–10). The relationship is shown in Figure 1.

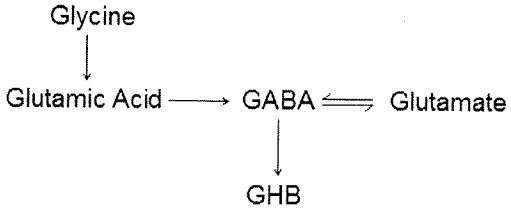


Figure 1. *GABA-Glutamate Cycle*

GABA is a normal, non-essential amino acid that does not readily cross the blood brain barrier. It is produced in peripheral body tissues and is also a normal constituent of certain plant material (12, 13). GABA has a higher blood brain transport threshold than essential amino but will cross the blood brain barrier at elevated plasma concentrations that occur in drug administrations (14, 15). Original studies (16) reported that GABA did not cross the blood brain barrier but subsequent work demonstrated that it does. Absorption of GABA is problematic so a number of more lipophilic drugs such citalopram, fluoxetine, benzodiazepines, and barbiturates are used to alter GABA brain concentrations (17–19).

GABA has become increasingly popular as a depressant drug in performance show horses. Many reports have been provided to the United States Equestrian Federation of its blatant abuse. GABA is available through a number of compounding pharmacies for this purpose. Its use in horse racing is not reported to be as widespread. The source of GABA used in this study was from a compounding pharmacy.

This paper presents a LC/MS method for the determination of GABA in horse plasma as the propyl chloroformate derivative. Husek (20, 21) have reported on the use of chloroformate as a general derivatizing agent and also for derivatizing amino acids for gas chromatography/mass spectrometry. Comparison of different amino acid derivatives have been reported by Uutela *et al* (22). Kasper (23) has reported on the use of propyl chloroformate and compared LC/MS/MS and GC/MS.

A commercially available amino acid analysis kit was used in this work based on propyl chloroformate derivatives. The extraction, derivatization, and sample concentration are done in a single tube in the aqueous phase.

Determination of a plasma threshold value for GABA in a normal horse population and its relationship to plasma concentrations from experimental drug administrations is presented.

MATERIALS AND METHODS

For population studies to determine plasma threshold values, 100 thoroughbred horses from a New York race track were sampled in a secure detention area prior to racing. Ten ml vacutainer tubes with sodium heparin as anticoagulant were used and refrigerated upon collection. Plasma samples were analyzed within 72 hrs of collection. In a second study, 12 pleasure horses of mixed breeds known not to have been administered GABA were used.

Experimental drug administration trials used six thoroughbred horses ranging between six and 10 years of age that were in excellent health. The animals were housed and treated in accordance with USDA regulations. During the trials, the horses were kept in box stalls and otherwise kept on pasture.

Carolina Gold was used as the source of GABA. The ingredients are shown in Table 1. Ten ml of the injectable solution was administered to each horse via the intra muscular route. Blood samples were collected immediately prior to injection and at 2, 4, 6, 8, 10, and 12 hours post injection.

Table 1.

Carolina Gold
Wedgewood Pharmacy

Ingredients:

• Gamma aminobutyric acid	165 mg/ml
• D- Phenylalanine	7.5 mg/ml
• L- Isoleucine	10 mg/ml
• L-Arginine	10 mg/ml
• L-Tryptophan	10 mg/ml
• Benzyl alcohol (preservative)	

Extraction and Derivatization

Liquid chromatography/mass spectrometry combined with the formation of propyl chloroformate derivatives using the Phenomenex EZ:faast Physiological Amino acid Analysis

LC/MS Kit was used (24). The Kit was used as recommended by the manufacturer with minor modifications. In brief, 100 ul of plasma was added to the pipette tips containing a strong cation exchange resin. Free amino acids were selectively extracted from plasma proteins that were removed with an acid wash and 30% propanol in water wash. Amino acids were eluted with the elution reagent into 2.0 ml polypropylene centrifuge tubes and derivatized with the propyl chloroformate reagent. Isooctane was used to extract the derivatized amino acids in the centrifuge tubes. Centrifugation was used to increase phase separation rather than direct transfer to glass vials supplied with the kit. The isooctane (100 µl) was transferred and concentrated to dryness in a glass concentration vial under a flow of nitrogen at room temperature. The residue was reconstituted in 100 µl of mobile phase in auto sampler vials containing reduced volume glass inserts. A schematic representation is shown in Figure 2. Methionine-d3 was used as the internal standard.

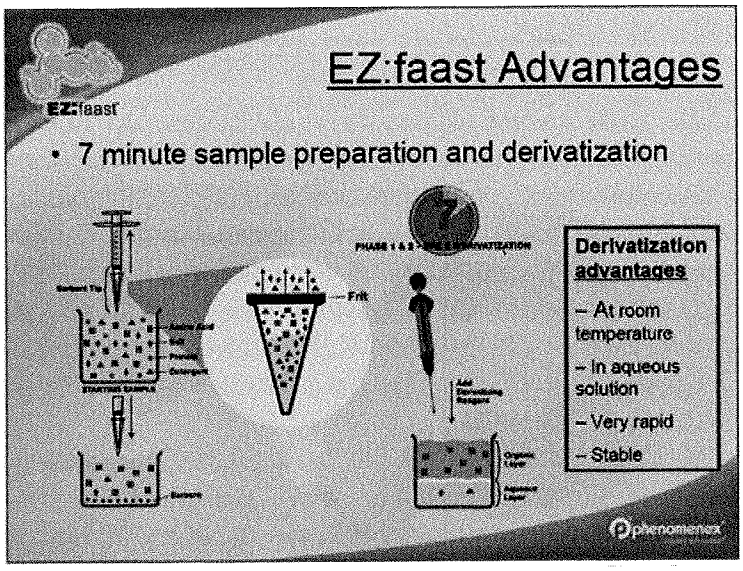


Figure 2.

Liquid Chromatography/Mass Spectrometry

An Agilent 1100 liquid chromatograph equipped with a degasser, binary pump, and auto sampler was used. The column was a Phenomenex EZ:faast AAA MS, 250 x 3.0 mm operated at 35°C in the gradient mode. The binary mobile phase was A: 10 mM ammonium formate in water and B: 10 mM ammonium formate in methanol. The gradient was 68% B for 0–13 minutes, then 100% 13–13.01 minutes and 13.01–17 minutes with a 3 minute equilibration time before restarting another gradient. One µl injections were made. An Agilent LC/MSD LS Mass Spectrometer was used in the positive ion mode. Operating conditions were: scan range 100–500 m/z, drying gas 13 L/minute, nebulizer pressure 60 psig, drying gas 300°C, and capillary voltage 2500 volts.

RESULTS

The structure and *derivatization* reaction for GABA propyl chloroformate is shown in Figure 3. It is a stable derivative that is sensitive to electrospray ionization. The derivative is separated

from alpha and beta aminobutyric acids and approximately 50 other endogenous amino acids in equine plasma samples as shown in Figure 4. The total ion chromatogram shown in Figure 5 demonstrates the complexity of the chromatogram of extracted and derivatized amino acids in normal horse plasma. Quantitative estimates were based on the internal standard, methionine-d3, supplied with and used according to the Kit instructions.

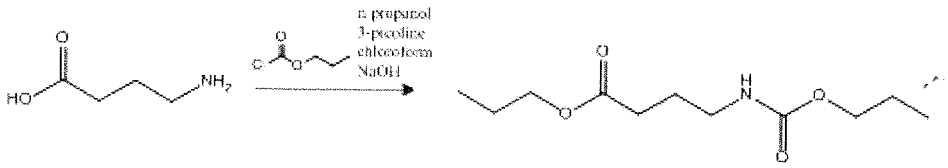


Figure 3. *GABA Propyl Chloroformate Derivatization*

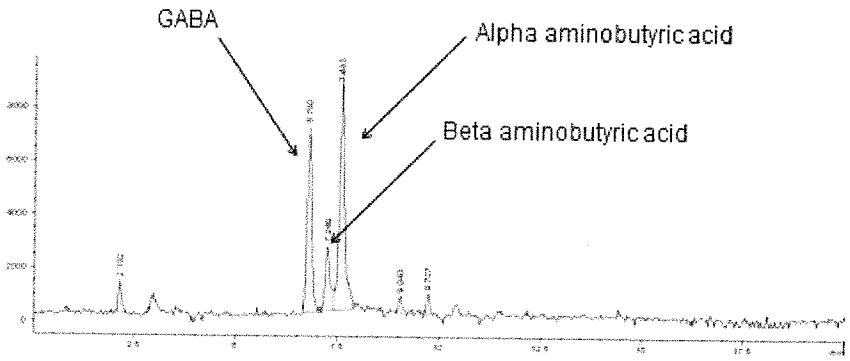


Figure 4. *EIC: GABA 2 Hour post-administration plasma sample*

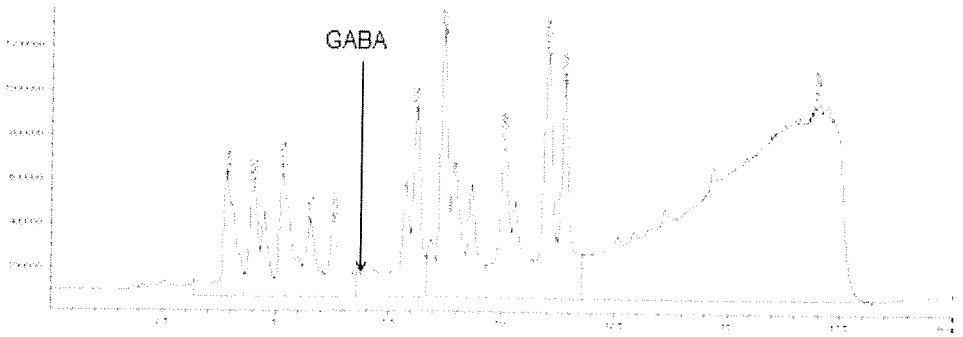
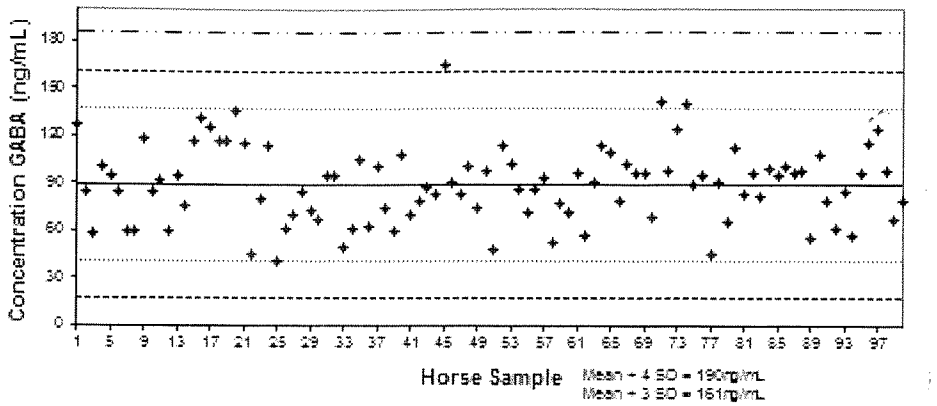


Figure 5. *TIC: GABA 2 Hour post-administration plasma sample*

The population survey of GABA plasma concentrations in 100 thoroughbred race horses is shown in Figure 6 including the mean ± 4 standard deviations. The data is normally distributed as shown in Graph 1 according to the Shapiro-Wilk test. Based on these data, a threshold plasma concentration value of 190 ng/ml of GABA has been established.



Graph 1. *Plasma GABA Concentrations in Race Horses*

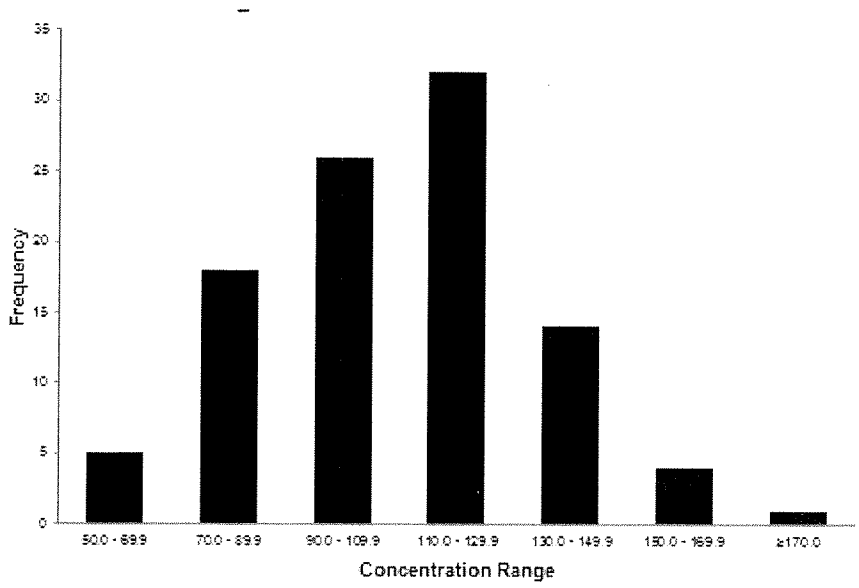


Figure 6. *Histogram—GABA in Horse Plasma*

The GABA plasma concentrations observed during the administration study of six horses exceeded 190 ng/ml in all study horses from two to six hours post-administration. The concentrations were below this level in all horses by 12 hours post-administration. This is shown in Figure 7.

A population survey of 12 pleasure horses is shown in Figure 8.

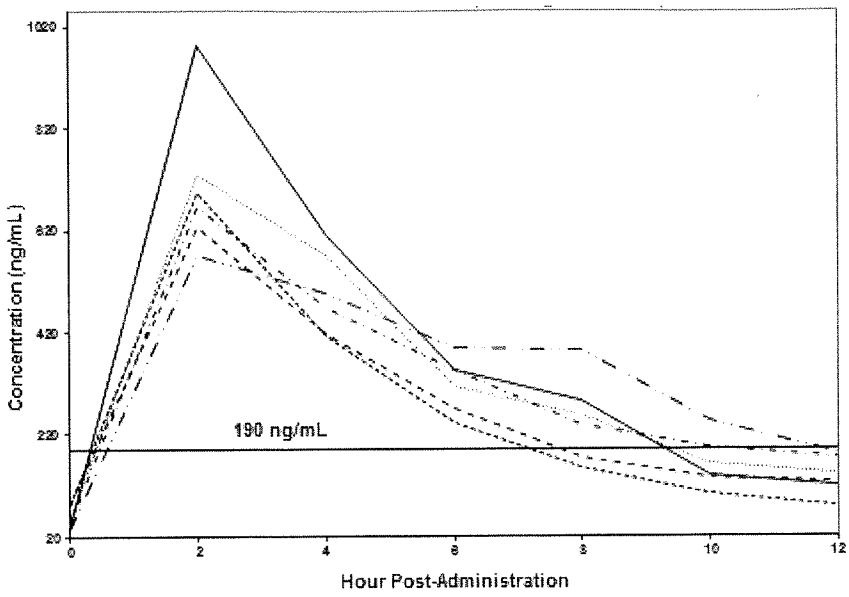


Figure 7. *Plasma GABA Concentrations in Horses Administered Carolina Gold (1.65 grams IM).*

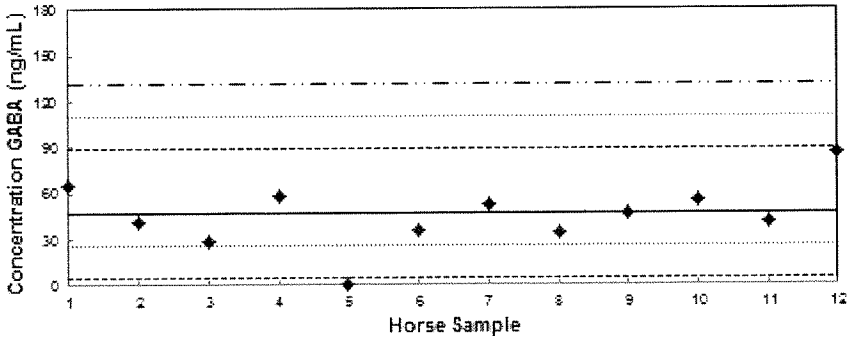


Figure 8. *Plasma GABA Concentrations in Pleasure Horses.*

Due to the small sample size a statistical analysis was not done. It is apparent, however, that GABA plasma concentrations in pleasure horses exposed to little or no exercise are less than half that of fit race horses. The reason for this difference is unknown. In addition to determination of GABA in horse plasma, the described test provides concentrations of essential free amino acids determined by alternative methods as shown in Table 2.

Amino Acid	Literature (mmol/L)	NYDRTP (mmol/L)
Phenylalanine	0.15	0.07
Tryptophan	0.18	0.09
Valine	0.30	0.27
Threonine	0.20	0.14
Lysine	0.22	0.16

*DePew, C.L. et al, "Changes in Concentrations of Hormones, Metabolites, and Amino Acids in Plasma of Adult Horses Relative to Overnight Feed Deprivation Followed by a Pellet-Hay Meal Fed at Noon". J. Anim. Sci. 1994. 72:1530-39.

Table 2. *Comparison of Amino Acid Contrations to Approximate Concentrations listed in Literature.**

CONCLUSION

The Phenomenex EZ:fast Physiological Amino Acid Kit provides a ready-to-use method for the determination of GABA and approximately 50 free amino acids in horse plasma. A single quadrupole LC/MS provides required sensitivity and selectivity for screening a large number of samples. Confirmation for forensic cases by tandem mass spectrometry is recommended. A GABA threshold plasma concentration of 190 ng/ml has been established based on the mean ± 4 standard deviations.

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Phenomenex Inc. EZ;faast LC/MS Free (Physiological) amino Acid Analysis Kit.