Journal of Chromatography, 493 (1989) 182–187
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4811

Note

Applicability of capillary gas chromatography to study caffeine distribution in the developing chicken egg

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(First received November 24th, 1988; revised manuscript received March 30th, 1989)

Caffeine is one of the most widely used drugs, consumed on a regular basis by most of the population from childhood onwards. A recent literature survey indicates that caffeine consumption is associated with a wide variety of diseases [1], detrimental effects on somatic and psychological health [2], growth retardation in term newborns [3] and spontaneous abortion [4]. It has been demonstrated in our laboratory that caffeine injected into three- or four-day-old chicken embryos induces a decrease in blood flow in the embryonic heart [5] and a dose-dependent increase in the frequency of cardiac malformations [6]. The quantitation and distribution of caffeine at different time intervals in the egg white is important for relating concentration effects of caffeine to cardiac defects in the chicken embryo.

Numerous gas chromatographic (GC) methods have been reported for the determination of caffeine, primarily for urine and serum samples obtained from a variety of species. One of our objectives was to optimize quantitation of caffeine in the presence of a different matrix, chicken egg white, by modifying existing techniques.

The aims of the present paper are to describe a simple and reproducible fused-silica capillary GC flame ionization detection (FID) method for the quantitation of caffeine in the presence of chicken egg white and to demonstrate its applicability for studying the distribution of caffeine in egg white as a function of time.

0378-4347/89/$03.50

EXPERIMENTAL

Materials

Caffeine (Sigma, St. Louis, MO, U.S.A.) and ketamine hydrochloride (Ketalar®, Parke-Davis, Morris Plains, NJ, U.S.A.) were used as reference standards. Ethyl acetate was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). An aqueous solution of 1 *M* sodium hydroxide was prepared from ACS reagent-grade chemicals (J.T. Baker, Phillipsburg, NJ, U.S.A.). Deionized, distilled water was used in the preparation of stock solutions throughout the analytical protocol. White Leghorn chicken eggs were obtained from the Department of Poultry Science at Colorado State University (Fort Collins, CO, U.S.A.) and stored for no longer than three days at 5°C.

Sample collection

Eggs were incubated in a forced-air, redwood, electric cabinet at 37–38.5°C with a relative humidity of 65–75%. At 80 h of incubation, eggs were windowed as described previously [7]. Through the observation window, a total volume of 1.0 ml Chick Ringer's saline (123 m*M* NaCl, 5 m*M* KCl, 1.6 m*M* $CaCl_2$) containing 3.5 mg caffeine was administered topically to the extraembryonic membranes of developing chick embryos. At 0, 2, 4, 8, 12 and 24 h after treatment, a 0.5-ml sample of egg white was removed from the pointed end of each egg and prepared for high-resolution fused-silica capillary GC.

Stock solution

Caffeine (115 mg/ml, equivalent to base) and the internal standard, ketamine hydrochloride (5 mg/ml), were prepared by dissolving these compounds in double-distilled water. The solutions were refrigerated for up to two months.

Instrumentation and chromatographic conditions

A Model 5890A Hewlett-Packard (Hewlett-Packard, Avondale, PA, U.S.A.) gas chromatograph equipped with a flame ionization detector and a fused-silica capillary column inlet system was used for all analyses. A Model 3393 AHP integrator was utilized for peak area counts.

A 5 m×0.53 mm I.D. cross-linked fused-silica capillary column (methyl silicone, film thickness 2.56 μm, Hewlett-Packard) was used in the analysis. The splitless injection mode employing a fused-silica insert (78 mm×2 mm I.D.) with a purge-off time of 1 min was used for 2-μl samples. The operating conditions for analysis were: injection port temperature, 225°C; initial oven (column) temperature, 160°C; initial hold time, 0 min; programming rate 1, 6°C/min; final oven temperature 1, 184°C; programming rate 2, 30°C/min; final oven temperature 2, 275°C; FID temperature, 325°C; carrier gas (helium) flow-rate, 15 ml/min; make-up gas (helium) flow-rate, 10 ml/min; septum purge flow-rate, 4 ml/min; hydrogen/air flow-rate (ml/min) ratio, 30:400.

Extraction

From each egg white sample, 0.09 ml was transferred into a clean 15-ml glass tube. To each tube were added the following: 0.5 ml of internal standard (5 mg/ml ketamine hydrochloride), 1 ml of double-distilled water, two drops of 1 *M* sodium hydroxide (to liberate free caffeine and ketamine bases and to precipitate proteins from egg white) and 1 ml of ethyl acetate. Tubes were capped and the aqueous phase was extracted by shaking the mixture for 10 min on a rocking shaker (Labquake® shaker, Model T400-110; Lab Industries, Berkeley, CA, U.S.A.). The samples were then centrifuged for 10 min with a clinical centrifuge. The organic layer (0.7–0.8 ml) was transferred to a clean 15-ml glass tube and dried in a 40°C water bath under a stream of nitrogen. The residue was reconstituted to 100 μl with ethyl acetate. The sample was mixed on a vortex mixer for 10 s and a 2-μl aliquot from each sample was injected into the gas chromatograph for analysis. Each egg white sample was extracted in duplicate.

Preparation of the standard curve

A 0.09-ml sample of blank egg white was spiked with varying volumes of aqueous caffeine solution (0.05–1.0 ml of 4 mg/ml caffeine), and the aqueous phase was adjusted to 1.90 ml with double-distilled water. The addition of internal standard, protein precipitant, and further sample treatment and extraction were carried out in a similar fashion to the analysis of unknown egg white samples. Samples for each caffeine concentration were prepared in duplicate.

Statistics

The Student *t*-test for paired samples (two-tailed) with a significance level of $p = 0.05$ was employed for the analysis of data.

RESULTS

Representative GC profiles obtained from an egg white blank and a sample taken from a caffeine-injected chicken egg are shown in Fig. 1. Extraneous peaks from endogenous egg white constituents were negligible and did not interfere with the analysis. Peaks with retention times of 2.6 and 3.1 min were identified as caffeine and ketamine, respectively. Data from a representative standard curve used in the quantitation of caffeine in egg white are presented in Table I.

Caffeine distribution results from this study are shown in Table II. A maximum mean concentration of 84.70 ± 17.96 μg/ml caffeine was observed in the egg white 24 h post-treatment. Mean concentrations of caffeine at 2, 4, 8, 12 and 24 h were significantly different ($p < 0.05$) from each other (except 12 h versus 24 h) and increased progressively with time. The range of caffeine values was 0.2–116.7 μg/ml.

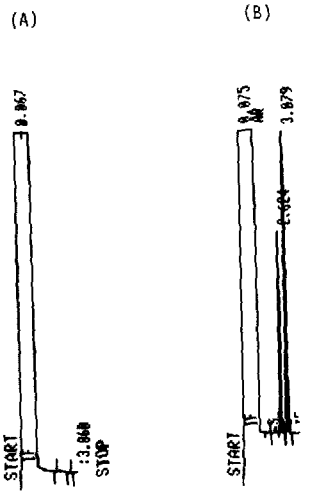

Fig. 1. Representative capillary gas chromatograms obtained from blank chicken egg white (A) and an egg white sample from a chicken egg (B) injected with 1 ml of Chick Ringer saline containing 3.5 mg of caffeine. The egg white sample was spiked with the internal standard, ketamine. The retention time for caffeine was 2.6 min and that of ketamine 3.1 min. The x-axis represents time in minutes.

TABLE I

CALIBRATION CURVE DATA FOR CHICKEN EGG WHITE

Linear regression line for caffeine: $y=5.62x-0.0339$, $r^2=0.9998$.

Amount of caffeine spiked (μg)	Caffeine/ketamine area ratio[a] (mean ± 1 S.D.)	Coefficient of variation (%)
0.2	0.037 ± 0.005	12.2
0.4	0.075 ± 0.006	8.2
0.8	0.154 ± 0.011	7.4
1.6	0.295 ± 0.007	2.6
2.4	0.434 ± 0.029	6.3
3.2	0.572 ± 0.014	2.4
4.0	0.718 ± 0.017	2.8

[a]Three samples, each injected twice.

TABLE II

DISTRIBUTION OF CAFFEINE IN THE DEVELOPING CHICKEN EGG

Sampling time (h)	Caffeine concentration (mean ± 1 S.D.) (μg/ml)
0	0
2	2.26 ± 1.79
4	13.83 ± 14.48
8	45.45 ± 17.13
12	61.92 ± 14.04
24	84.76 ± 17.96

DISCUSSION

Megabore fused-silica capillary columns have been extensively used in the quantitation of complex matrices. In the present study, we report a simple and fast one-step GC–FID method to study caffeine distribution in the developing chicken egg.

A splitless injection mode provided the required sensitivity that permitted analysis of caffeine in a small egg white sample (0.09 ml). This sample size enabled duplicate extraction and multiple sampling of egg white at various times from the same egg. Two drops of 1 *M* sodium hydroxide were sufficient for protein precipitation and liberation of respective free bases without diluting the samples. Recovery of spiked caffeine and ketamine from blank egg white sample (0.09 ml) as compared to that from distilled water (0.09 ml) was more than 98%. Nevertheless, a 0.09-ml sample of blank egg white was added in each tube during the preparation of standard curve in order to mimic the matrix of egg white samples.

A multi-step temperature ramping resulted in improved chromatographic peak shapes for caffeine and the internal standard, ketamine. A relatively short run time of 3 min was obtained without interference from endogenous egg white substances (Fig. 1). Column conditioning by increasing oven temperature to 275°C after every five sample injections permitted analysis of more than 100 samples with no peak tailing or carry-over of endogenous substances.

The optimization of assay technique has been found to show good linearity over the concentration range of caffeine studied (0.2–4 μg/ml, Table I). The line of best fit was described by $y = 5.62x + 0.0339$ with a coefficient of determination (r^2) of 0.9998. Within-run precision of a representative calibration curve demonstrated good reproducibility with the coefficient of variation ranging from 2.4 to 12.2% (Table I). The refrigerated samples were stable for at least seven days, showing excellent within- and between-day reproducibility over the entire concentration range.

Our results show that the distribution of caffeine (administered topically) is a relatively slow process (Table II) and is a function of time. It takes at least 24 h for injected caffeine to diffuse through the yolk into the egg white. This slow distribution of caffeine across the egg may explain, in part, the (wide) variation in its cardioteratogenicity observed in the chick embryo [6]. Therefore, sampling time and quantitation of caffeine and other teratogens in the developing chicken embryo should be considered in the experimental design of such studies to gain a better insight into concentration–effect relationships.

ACKNOWLEDGEMENTS

The authors wish to extend their appreciation to the University of Wyoming College of Health Sciences for financial support of this project. We would like to thank Mrs. Carol Persson for her superb secretarial assistance in the preparation of this manuscript.

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