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Note

Comparison of gas–liquid chromatography with nitrogen–phosphorus selective detection and high-performance liquid chromatography methods for caffeine determination in plasma and tissues

M. BONATI, D. CASTELLI, R. LATINI and S. GARATTINI

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

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Highly reliable analytical methods for the determination of caffeine in various biological materials are required to provide data needed to elucidate the role of caffeine in clinical therapeutics and toxicology [1—6]. Gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) have been used extensively in our and in other laboratories [7—12]. This report describes experience gained with a programme of quality control which enabled us to check the precision and the comparability of the two techniques.

MATERIALS AND METHODS

Standard and reagents

Anhydrous caffeine and theophylline monohydrate were obtained from Carlo Erba, Milan, Italy. Hexane, chloroform, methanol and acetonitrile (Lichrosolv, Merck, Darmstadt, G.F.R.) were UV grade. Tetrahexylammonium hydroxide was purchased from Hässle, Göteborg, Sweden. Standard solution: caffeine was dissolved in methanol (100 μg/ml) and stored at +4°.

The theophylline-1-propyl derivative, the internal standard, was prepared as follows. Theophylline (50—100 μg) was dissolved in 2 ml of 0.1 *N* sodium hydroxide and 0.5 ml of a solution of 0.1 *N* tetrahexylammonium hydroxide in 0.1 *N* sodium hydroxide. Three ml of methylene chloride—1-iodopropane (5:1) were added to the aqueous phase and the samples were horizontally shaken in glass tubes for 40 min in a thermostatic water bath at 60°. After centrifugation the organic layer was transferred into another glass tube and dried under nitrogen. Two ml of hexane were added to the dry residue, left for 10 min in an ultrasonic tube and dried. The dry residue was dissolved in hexane in order to reach a concentration of theophylline derivative of 10 μg/ml (stability at 4° up to 4 weeks).

This internal standard was chosen because of its retention time for both methods, its chemical similarity with caffeine and its difference from possibly interfering substances.

Gas chromatograph—nitrogen—phosphorus selective detector

A Carlo Erba Fractovap Model 2003 gas chromatograph equipped with a nitrogen–phosphorus selective detector with potassium chloride as alkaline ion source was used. The column was a glass tube (3 m × 3 mm I.D.) packed with Gas-Chrom Q (100—120 mesh) coated with 5% SE-30 (Carlo Erba) and conditioned for 24 h at 290°. Operating conditions were as follows: helium flow-rate (carrier), 40 ml/min; hydrogen flow-rate, 35 ml/min; air flow-rate, 300 ml/min; injection port temperature 275°; and column temperature 270°.

High-performance liquid chromatograph

A Perkin Elmer series 2/2 liquid chromatograph equipped with a Model LC 55 detector, and a reversed-phase column (0.25 m × 2.6 mm) packed with 10 μm (average particle diameter) octadecylsilica (ODS-SIL-X-1; Perkin Elmer, Norwalk, Conn., U.S.A.) was used. The column was eluted with a mixture of 750 ml of twice-distilled water and 250 ml of acetonitrile. The flow-rate was 1.2 ml/min. The detector was used at 273 nm.

Extraction

A 0.1—1.0-ml sample of plasma or brain homogenate (1:10 w/v of distilled water) is brought to pH 7.0 with 1.9—1.0 ml of $Na_2B_4O_7 \cdot 10\ H_2O$, 0.08 *M* buffer. The samples are shaken for 20 min with 5 ml of chloroform in glass test tubes containing 0.05 ml of the internal standard solution.

After centrifugation for 10 min at 1200 *g*, 4 ml of the chloroform mixture are transferred into another test tube and evaporated to dryness under a gentle stream of nitrogen in a thermostatic water bath at 40°. The residue is dissolved in 50 μl of methanol and 1—2 μl of this solution are injected into the gas chromatograph.

The calibration curve is prepared with increasing amounts, 5—60 μl, of the methanol solution of caffeine. To the dry residue, 1 ml of blank plasma or blank brain homogenate is added, and the samples are then processed as above. After gas chromatographic determination the methanol solvent is evaporated. The residue is dissolved in 100 μl of the chromatographic mobile phase (see above) and 10 μl of each sample are injected into the liquid chromatograph. The average recovery from plasma was 92% and from brain 86%.

Experimental design

Brain and plasma samples which during a toxicological study gave caffeine levels of 4.7—5.3 μg/ml (or μg/g) and of 47—53 μg/ml (or μg/g) were pooled to provide the material for the quality control. Each pool of brain and plasma was divided into five specimens. The four blocks of samples thus obtained were analysed over a period of three months for a total of five replicates for each brain sample and six for each plasma sample. The volumes extracted were the following: plasma: 0.1 ml (low concentration), 0.05 (high concentration); brian: 1.0 ml (low concentration), 0.5 (high concentration). A calibra-

tion curve (0.5, 1.0, 2.0, 3.0, 4.0, 6.0 μg of caffeine) was made for each run of plasma and brain homogenate samples. Extractions and measurements were carried out by a team of three operators.

Statistical analysis

The precision of the two methods was calculated from the coefficient of variation (CV%) (standard deviation/mean·100). GLC—nitrogen—phosphorus selective detection (NPSD) and HPLC methods were first compared according to Westgard and Hunt [13] by linear regression analysis, and then more correctly by the method of Bartlett [14] applied to the natural logarithms of measured concentrations [15, 16].

RESULTS

Fig. 1 shows typical GLC—NPSD and HPLC chromatograms. The overall sensitivity of the methods was 0.25 μg/ml; using more than 1 ml of plasma lower concentrations can be measured, also increasing the volume of the extractive solvent. Internal and external calibration curves (Fig. 2) passed through the origin and were linear from 0.25 to 2 μg for GLC—NPSD and from 0.25 to more than 20 μg for HPLC. Caffeine concentrations exceeding the range of linearity of the GLC calibration curve were obtained from graphical interpolation. Table I shows coefficients of variation given by the two methods. Coefficients of variation for plasma and brain did not differ significantly, but they were higher with the GLC method at the lower concentration.

The GLC—NPSD method was chosen as reference (for the linear regression analysis on natural values) since it was employed for two years in this laboratory before the introduction of the HPLC technique. This first approach gave:

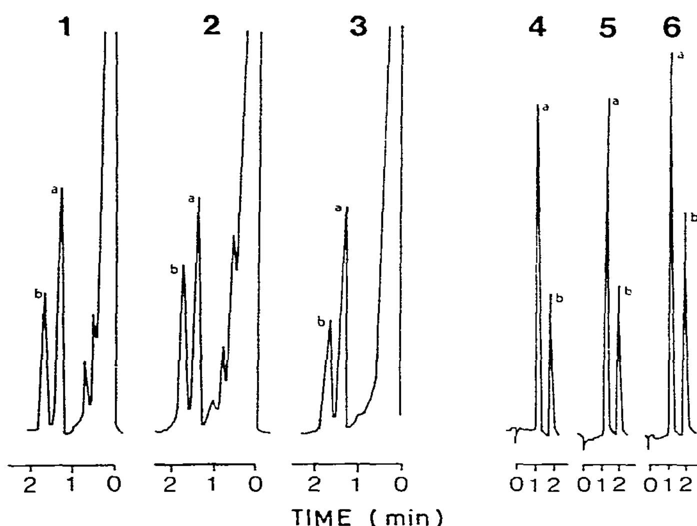

Fig. 1. Gas chromatograms of 1: plasma extract sample; 2: brain extract sample; 3: external sample. HPLC chromatograms of 4: external sample; 5: brain extract sample; 6: plasma extract sample. Peaks: a = caffeine; b = theophylline-1-propyl derivative.

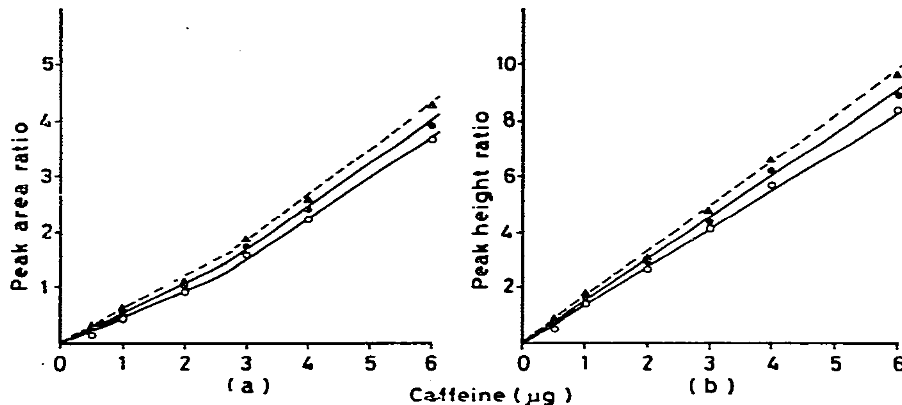

Fig. 2. Standard calibration curves for caffeine by (a) GLC—NPSD and (b) HPLC methods. ▲——▲, External standard; ●——●, internal standard, from plasma; ○——○, from brain. Propyl-theophylline was added just before injecting the sample, in order to estimate absolute recovery.

TABLE I

PRECISION OF CAFFEINE ASSAY METHODS IN PLASMA AND BRAIN

	Plasma				Brain			
	GLC—NPSD		HPLC		GLC—NPSD		HPLC	
	Low	High	Low	High	Low	High	Low	High
X	4.8	44.0	4.8	41.2	5.0	45.5	5.2	46.2
S.D.	0.7	4.5	0.5	4.1	0.9	3.5	0.5	4.3
CV	14.6	10.2	10.4	10.0	18.0	7.7	9.6	9.3
n	30	30	30	30	25	25	25	25

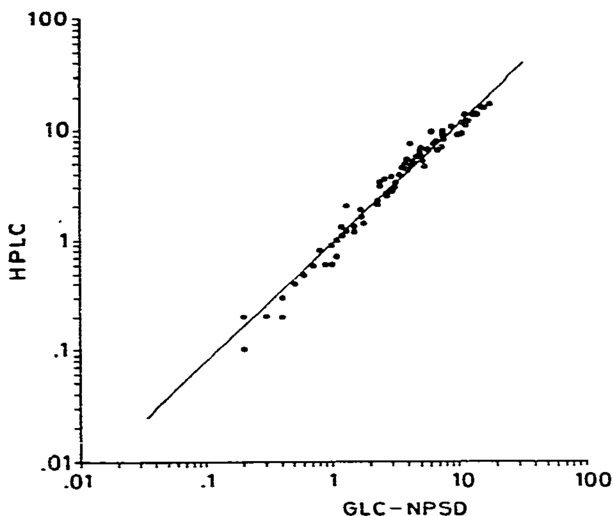

Fig. 3. Comparison between GLC—NPSD and HPLC assays of 88 plasma rat samples.

$r = 0.982$ ($p \ll 0.01$) and $F = 2233$ ($p \ll 0.01$) for a line with a slope of 1.04 and an intercept of 0.209, not significantly different from zero.

As the true caffeine concentrations were not available the accuracy of the reference method could not be established, and since both methods did not show a constant variance over the entire concentration range (constant CV, see Table I) we applied the Bartlett approach to the ln-transformed data. This more correct approach gave $r = 0.988$ and $F = 3533$, both highly significant, for a line with a slope of 1.13 (see also Fig. 3).

If we assume a concentration of 10 µg/ml (chosen as representative of the data in Fig. 3) for the GLC—NPSD method, the corresponding value found using HPLC is 10.76 ± 1.48 µg/ml.

DISCUSSION

In this laboratory these two methods responded as reported in the original papers [9, 10], except that the HPLC detector gave a linear response over a broader range of concentrations than the NPSD detector. From the present study both methods appeared to be sufficiently reliable, although lower concentrations were better assayed with HPLC, as shown by the lower coefficient of variation at the low concentration (Table I).

Toxicological studies deal with very low concentrations of caffeine in humans and with doses and concentrations in animals of up to a hundred times greater [17]. Because of its greater precision over a wider range of concentrations and because it measures caffeine metabolites as well, HPLC appears to be the method of choice.

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