Journal of Chromatography, 162 (1979) 223—228
Biomedical Applications
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CHROMBIO. 248

CAFFEINE DETERMINATION IN RAT PLASMA

A COMPARATIVE STUDY OF MICROMETHODS

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SUMMARY

Confronted with the need for a sensitive (1 mg/l), specific and reproducible (<5%) method for the determination of caffeine in small plasma samples (10—50 μl) of small laboratory animals, two existing methods, radioactive labelling and gas chromatography, were adapted. The first step, common to both methods, is chloroform extraction, followed by either gas-chromatographic analysis or radioactivity measurement. These methods were compared by using a common internal standard, labelled caffeine, measured before and after the extraction step. The initial requirements were fulfilled and the correlation of the results proved to be excellent.

These methods can be extended to animals other than the rat, and to organs or biological fluids other than plasma. The very small amount of plasma needed for the determination allows pharmacokinetic studies to be conducted in small laboratory animals. Further, by combining the two methods it is possible to perform rather complex investigations, such as evaluating the extent of caffeine metabolism and, at the same time, determining levels in the case of chronic administration.

INTRODUCTION

Several methods have been proposed for the determination of caffeine in human blood: after extraction by means of an organic solvent (generally benzene or chloroform), caffeine is measured by UV spectrophotometry [1—4] or by gas chromatography (GC) [5,6]. Sensitivity and specificity are two major problems in this type of determination. Sensitivity has been gained by using large plasma samples (>2 ml) whose extracts are concentrated. Plasma levels of 1—2 mg/l could be determined in this manner. Specificity has been obtained by column separation in the case of GC and tentatively by subtracting blank values when using UV absorption. Apart from the high variability of the blank values observed, such a procedure does not differentiate caffeine from various

endogenous or exogenous absorbing compounds. Routh [7] proposed a differential method to compensate for the absorption due to the presence of theophylline and theobromine, two important metabolites of caffeine. Study of the metabolic pathway of caffeine [8—12], however, has revealed the presence of a great variety of metabolites which are being investigated in the case of chronic caffeine administration [13]. More recently, radioimmunoassay [14] high-pressure liquid chromatography [14,15], and thin-layer chromatography (TLC)—densitometry [16] have been proposed for the measurement of caffeine in the plasma.

A potential major draw-back of these methods is that none of them utilizes a real internal standard to control the whole procedure of extraction, quantitation and reproducibility.

For small laboratory animals (such as rats and mice), the plasma volume required is too large for the above methods to be applicable. Isotopic labelling has been used mainly for metabolic studies [9, 10, 12, 17] or for investigations of transfer processes [18, 19]. In the present investigation it was our intention to adapt two existing methods, isotopic labelling and GC, for small samples (10—50 μl) and to correlate the results by using a common internal standard, [1-CH_3-^{14}C]caffeine, which would in addition allow us to evaluate the recovery over the whole procedure.

EXPERIMENTAL

Biological samples

Adult Sprague-Dawley male and female rats, weighing 200—300 g, were used. All the animals were fasted for one night before caffeine administration.

Pure aqueous or NaCl isotonic solutions of caffeine benzoate were labelled with (1-CH_3-^{14}C)caffeine (New England Nuclear, Boston, Mass., U.S.A.). Caffeine solutions were administered either orally to unanaesthetized animals or intravenously to rats under ether narcosis. Blood was collected in heparinized tubes under ether anaesthesia by aortic puncture. Plasma samples were frozen until analysis, for a time period which never exceeded four weeks. Urine samples were taken from some animals several hours after caffeine administration. The dose of caffeine varied from 0.10 to 50 mg/kg body wt. The rats were generally given 5 μCi, and exceptionally up to 50 μCi. The time interval between administration and blood collection was either 2 min or between 0.5 and 8 h. We assumed that, in plasma, 2 min after i.v. administration, the concentration of the caffeine metabolites is negligible and that the activity measured is essentially due to caffeine alone.

Radioactivity measurements

Radioactivity was measured directly in 10-μl plasma samples and in chloroform extracts of these samples. For simple counting, 10 μl plasma were extracted in 500 μl chloroform; for TLC analysis, 250 μl plasma were extracted in 800 μl chloroform. All the extractions took place in the presence of a buffer (pH 10 glycine buffer according to Sörensen) and chloroform was always evaporated. All the samples were dissolved in Soluene-100 (Packard), except for TLC studies where the residues after chloroform evaporation were dissolved in 0.05 *N* HCl.

TLC was performed on silica-gel plates (Merck No. 5737) with chloroform—ethanol (9:1) or acetone—*n*-butanol—chloroform—ammonia 25% (30:40:30:10) as solvent mixtures for one- or two-dimensional migration, respectively [20]. After separation, a radiographic film (Kodak Kodirex) was placed against the plate for 1—4 weeks. The silica gel spots located on the film were scraped from the plate and their activities were counted. The spots were identified by means of labelled standards applied in parallel.

The samples were counted in a spectrometer (TriCarb, Packard), after addition of a standard PPO—POPOP—toluol—ethanol scintillator. Quenching was controlled by channel ratio and by external standardisation.

Gas chromatography

Most of the samples used in determinations of isotopic labelling were also analysed by GC using a slightly different extraction method. To 50-μl plasma samples in 0.3-ml conical vials, 5 μl citrate buffer (pH 6) and 50 μl chloroform were added. The contents of each vial were mixed for 30 sec and then centrifuged at 200 *g* for 10 min. Two μl were drawn from the chloroform layer under the lipoprotein interface and injected into a gas chromatograph (Perkin-Elmer 3920) equipped with a heated injection port, a glass column (1.8 m × 2 mm I.D.) packed with 3% SE-30 on Chromosorb W AW DMCS (80—100 mesh), and a flame ionization detector. The temperature conditions were 250° for the injector and the detector and 190° for the column. The carrier gas was N_2 plus HCOOH vapour at a flow-rate of 30 ml/min. No internal standard was used. Peaks were quantified (Hewlett-Packard 3380A integrator) by comparison with standard solutions covering a range corresponding to that of a given experiment, before and after each series.

RESULTS AND DISCUSSION

Benzene, toluene, and diethyl ether—chloroform and diethyl ether—acetone mixtures have also been used to extract caffeine from plasma. In our hands chloroform gave the best results in preliminary trials. By changing the pH value from 5 to 12, the recovery of caffeine remained unchanged. However, the pH value had a strong influence on the extraction of caffeine metabolites, either basic or acidic, as shown by TLC of extracts of urine samples. In order to study the demethylated metabolites of caffeine, the extractions for radioactive measurements were conducted at pH 10. On the other hand, in order to avoid the introduction of more polar molecules into the GC column, citrate buffer (pH 6) was used for the GC determinations.

When measuring caffeine by GC the polarity of the molecule provokes a loss of sensitivity and an asymmetrical form of the peak at low concentrations. Since the volatility of the caffeine is low, the column temperature needs to be high and the deactivation by silyl groups, generally used for this purpose, is not sufficient. We used a method proposed by Welton [21] for the analysis of barbiturates, which consists of adding formic acid vapour to the carrier gas. By applying this method, we could obtain symmetrical peaks, with no lengthening of the retention time, down to 0.5 ng caffeine in 1 μl of injected chloroform.

Another problem encountered in this type of determination is the use of an internal standard. A molecule chemically different from caffeine can not be considered as far as the extraction step is concerned, which is indeed the most critical one. Having already an isotopic internal standard for the over-all purpose of this study, we preferred external calibration for the GC determinations.

Statistics

All the experimental errors are expressed as the variation coefficient ($s/\bar{x}$) or as the ratio of the slope error over the slope (s_b/b).

Specificity

The specificity of the determinations was assessed in two different ways. Chloroform extracts, analysed by TLC, showed that if a single spot was to be attributed to caffeine 2 min after i.v. administration, two other spots could be detected after 1 h or more. The identification of caffeine was performed by two-dimensional TLC. Further, the product corresponding to the peak attributed to caffeine in the gas chromatograms has been identified as pure caffeine by mass spectrometry (MS) using a GC—MS method (LKB 2091; GC conditions identical to those used above, except for the use of pure helium as carrier gas).

Linearity and reproducibility

The linearity of both extraction and detection methods was verified by adding known amounts of caffeine, ranging from 1 to 25 mg/l, to rat plasma. Linear relationships were obtained by both methods ($r > 0.99$).

The reproducibility of each method was determined at different concentration levels (1—20 mg/l) by repeat extractions and determinations under identical conditions. In all cases, the reproducibility was found to be better than 5%.

Recovery

The assumption that labelled caffeine could be used as a common internal standard for both methods was verified by counting the total activity of a plasma sample, the total activity of its chloroform extract and the activity of the caffeine spot after TLC separation and scraping. The recovery of the activity of a caffeine standard spot compared with that of the solution before migration was found to be 90 ± 3%.

Therefore chloroform extracts from plasma samples collected 2 min after i.v. administration were applied to the TLC plates. The plates were divided into 10 strips (1-cm wide) which were scraped for measurement of their activity. The strips corresponding to the location of the caffeine standard spot contained 94 ± 6% ($n = 9$) of the total activity. When the time before collection of the samples was longer than 2 min, the activity of the extracts decreased to 67% after 1 h, 19% after 4 h and 1.8% after 8 h. Linear relationships were obtained by plotting the concentration of caffeine measured by radioactivity counting or by GC against the total activity of plasma collected 2 min after i.v. administration of different concentrations. Values for the slopes of the regression lines were 1.02 ± 0.02 ($r = 0.999$) for ^{14}C-labelling and 0.93 ± 0.02 ($r = 0.999$) for GC. Thereafter, values obtained by GC were corrected for by this recovery co-

efficient. The difference observed between the two methods was due to the fact that the solvent volume ratios were not the same, being 10:1 and 1:1, respectively.

Correlation between the methods

Both methods were correlated by measuring the caffeine content of 12 plasma samples collected more than 30 min after oral administration of solutions ranging from 1 to 50 mg/kg body wt. A value of 0.993 was obtained for the correlation coefficient (slope = 1.04 ± 0.04) (Fig. 1). Table I shows the main characteristics of the methods.

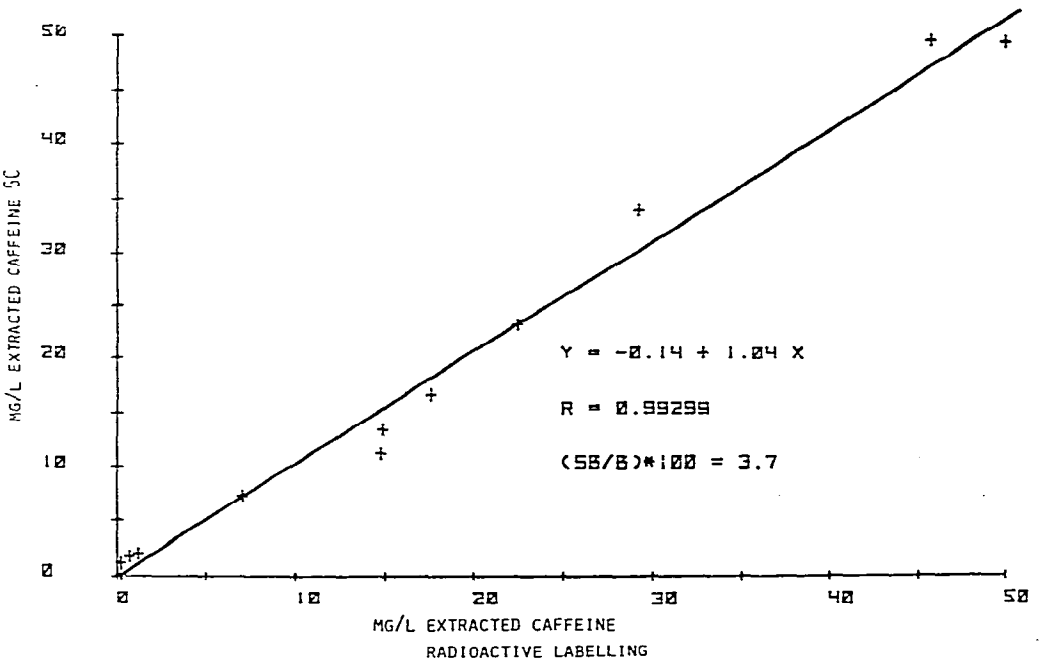

Fig. 1. Correlation between the methods.

CONCLUSIONS

We have adapted two existing methods, radioactive labelling and GC, for the determination of caffeine in very small plasma samples (10—50 μl). These methods were compared by using a common internal standard, labelled caffeine, measured before and after the extraction step. The correlation of the results proved to be excellent.

These methods can be extended to animals other than the rat, and to organs or biological fluids other than plasma. The very small amount of plasma needed for the determination allows one to conduct pharmacokinetic studies in small laboratory animals. Further, by combining the two methods, it is possible to perform complex investigations, such as obtaining an evaluation of the extent of caffeine metabolism, and, at the same time, determining levels in the case of chronic administration.

TABLE I

METHODS OF CAFFEINE DETERMINATION USED IN THIS WORK

	GC	^{14}C	^{14}C + TLC
Plasma (ml)	0.05	0.01	0.05
Reproducibility	5%	5%	5%
Sensitivity (mg/l)	0.1	According to specific activity	According to specific activity
Recovery	93%	100%	94%
Specificity:			
Substance	Caffeine	Caffeine + radioactive metabolites	Caffeine
Control	Mass spectrometry	TLC autoradiography	TLC autoradiography

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. H.P. Würzner for valuable discussions and Ms J. Markovic for skillful technical assistance.

REFERENCES

1 J. Axelrod and J. Reichenthal, J. Pharm. Ther., 107 (1953) 519.
2 G. Sant'Ambrogio, P. Mognoni and L. Ventrella, Arch. Int. Pharmacodyn., 150 (1964) 259.
3 V. Marks and J.F. Kelly, Lancet, i (1973) 827.
4 T. Chvasta and R. Cooke, Gastroenterology, 61 (1971) 838.
5 F.L. Grab and J.A. Reinstein, J. Pharm. Sci., 57 (1968) 1703.
6 T. Demas and B.E. Statland, Clin. Chem., 23 (1977) 1156.
7 J.I. Routh, N.A. Shane, E.G. Arredondo and W.D. Paul, Clin. Chem., 15 (1969) 661.
8 H.H. Cornish and A.A. Christman, J. Biol. Chem., 228 (1957) 315.
9 K.L. Khanna, G.S. Rao and H.H. Cornish, Toxicol. Appl. Pharmacol., 23 (1972) 720.
10 G.S. Rao, K.L. Khanna and H.H. Cornish, Experientia, 29 (1973) 953.
11 A.W. Burg and M. Stein, Biochem. Pharmacol., 21 (1957) 909.
12 M. Arnaud, Experientia, 32 (1976) 1238.
13 R.N. Warren, J. Chromatogr., 40 (1969) 468.
14 C.G. Cook, C.R. Tallent, E.W. Amerson, M.W. Myers, J.A. Kepler, G.F. Taylor and H.D. Christensen, J. Pharm. Exp. Ther., 199 (1976) 619.
15 G.W. Peng, M.A.F. Gadalla and W.L. Chiou, Clin. Chem., 24 (1978) 357.
16 W. Feldheim, E.H. Reimerdes and G.-R. Storm, Z. Lebensm.-Unters.-Forsch., 165 (1977) 204.
17 A.W. Burg and R. Werner, Biochem. Pharmacol., 21 (1972) 923.
18 B. Schmidt, Colloq. Int. Chim. Cafes, Trieste, 1967; ASIC, 1968, p. 286.
19 G. Czok, Colloq. Int. Chim. Cafes, Trieste, 1967; ASIC, 1968, p. 292.
20 E. Stahl, Dünnschicht-Chromatographie, Springer, Berlin, 1967.
21 B. Welton, Chromatographia, 3 (1970) 211.