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Simple and accurate determination of methylpyrazines in biofluids using high-performance liquid chromatography

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ABSTRACT

The determination of six methylpyrazines was performed using high-performance liquid chromatography (HPLC). Methylpyrazines were simultaneously extracted and injected onto a silica gel column with a syringe-type minicolumn packed with diatomaceous earth granules. The extraction–injection solvent used was dichloromethane and the mobile phase solvent for HPLC was dichloromethane containing 0.08% of 1.65 *M* ammonia solution and 0.5% of methanol. Methylpyrazines were detected using an ultraviolet detector set at 275 nm. Linear relationships between the amount of sample and peak height were confirmed from 50 ng/ml to 10 μg/ml of the biofluids. When an aliquot of 10 μl of biofluid was introduced to the minicolumn, the detection limit of methylpyrazines was as low as 30 ng/ml with each pyrazine derivative. The method is simple and accurate and is thus applicable to pharmacokinetic studies which are performed on animals. The results showed that the possible pharmacological effects of methylpyrazines might be evaluated pharmacokinetically using this newly developed technique.

INTRODUCTION

In 1962, tetramethylpyrazine (Fig. 1) was identified in cultures of a strain of *Bacillus subtilis* isolated in Japan from a traditional soybean food called natto [1]. Other methylpyrazines, including mono-, di- and trimethyl derivatives (Fig. 1),

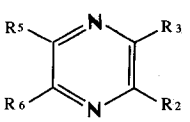

Fig. 1. Structure of methylpyrazines. Methyl substitutions on the pyrazine nucleus are suggested by the number and position of the substituent R.

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were also identified in human urine by head-space gas analysis using a gas chromatographic–mass spectrometric (GC–MS) method developed in the early 1970s [2]. In China, tetramethylpyrazine isolated from the Chinese medicinal plant *Cnidium rhizome* has been used clinically in patients suffering from conjestive heart failure [3,4]. This compound showed vasodilation [5] and anticoagulant *in vitro* activity [6]. In Nigeria, the same pyrazine derivative was isolated from an African plant, *Jatropha podagrica* [5,7,8]. Recent studies by Novotny and co-workers [9–12] clearly showed that 2,5-dimethylpyrazine is a physiological urinary element in mice and that it inhibits puberty in female mice. Pharmacological studies in Japan have shown that these methylpyrazines significantly delay the first vaginal oestrus in female rats [13]. Studies of methylpyrazines have so far been conducted under categories such as: (1) plant chemistry with the aim of discovering new drugs [3–8]; (2) physiology to resolve the role of pyrazines in female animals [9–12,14]; and (3) chemistry of nutrients in fermented foodstuffs, especially in Japan [1,15–17].

A GC–MS technique was used in these studies and no alternative technique has yet been reported. As methylpyrazines are very volatile, the condensation of organic solvent extracts is so difficult that many workers use head-space gas analysis without accurately determining methylpyrazines in the aqueous matrix. It is therefore very important to develop a new method which allows the simple and accurate determination of methylpyrazines. To overcome these difficulties, high-performance liquid chromatography combined with a direct injection technique using a syringe-type minicolumn packed with diatomaceous earth granules has been studied [18,19]. By retaining the aqueous sample solutions in the minicolumn, organic solvent extraction and injection can be performed simultaneously. This method has been used on plasma and urine samples obtained from rats assigned to pharmacokinetic model studies.

EXPERIMENTAL

Chemicals

Monomethylpyrazine, 2,3-, 2.5- and 2,6-dimethylpyrazines, trimethylpyrazine and tetramethylpyrazine were purchased from Aldrich (Milwaukee, WI, USA). Sulphuric acid, ammonia solution, sodium hydrogencarbonate and organic solvents donated by Wako (Osaka, Japan) were of analytical-reagent grade.

Apparatus

A syringe-type minicolumn (Extrashot) purchased from Kusano Scientific (Tokyo, Japan) was packed with 40 μl of diatomaceous earth granules (particle size 50–70 μm) which were pre-washed with a 5% sodium hydrogencarbonate solution. The HPLC apparatus consisted of a solvent delivery pump (BIP-I, Jasco, Tokyo, Japan), an ultraviolet detector (Uvidec 100-VI, Jasco), a syringe-loading sample injector (Rheodyne, Cotati, CA, USA) fitted with a 100-μl loop

and an integrator (Chromatocorder 12, System Instruments, Tokyo, Japan). The analytical column was a LiChrosorb Si-60 column, particle size 5 μm, column size 250 mm × 4 mm I.D. (Merck, Darmstadt, Germany).

Analytical procedures

The HPLC system was conditioned with a mobile phase solvent mixture of dichloromethane containing 0.08% of 1.65 *M* ammonia solution and 0.5% of methanol at a flow-rate of 1.0 ml/min at room temperature. An aliquot of 2 μl of the 1.65 *M* ammonia solution was loaded onto the surface of the packed material in the syringe-type minicolumn and then 10 μl of test solution (plasma, urine or authentic solutions of pyrazines in distilled water) were introduced. The minicolumn was then attached to the syringe-loading sample injector of the HPLC system. A 130-μl volume of dichloromethane was introduced as an extraction–injection solvent using a tuberculin test glass syringe for a period of 5–10 s. The ultraviolet detector was set at 275 nm and 0.0025–0.08 a.u.f.s. depending on sample concentrations.

Recovery and calibration

Recoveries of each methylpyrazine were estimated by the ratio of peak area data obtained with the minicolumn to those by direct injection of a known amount of solution in *n*-hexane.

Calibration was based on direct peak heights. The amount of sample was so small that the addition of an internal standard was neither possible nor necessary prior to analysis. Peak heights at various concentrations (0.05, 0.1, 0.5, 2.5 and 10 μg/ml) were examined for linearity to facilitate direct calibration and reproducibility.

Application to pharmacokinetic studies in rats

Three male Wister rats weighing 300–350 g were administered a saline solution of 2,5-dimethylpyrazine intraperitoneally. The pyrazine dose was 100 mg/kg body weight. Blood samples were collected from the tail veins with heparin-treated capillaries before administration and 1, 3, 6, 9, 12 and 24 h after administration. Plasma specimens were collected by centrifugation at 2000 *g* for 10 min. Urine samples were collected at baseline and timed samples followed the administration from 0 to 3, 3 to 6, 6 to 9, 9 to 12 and 12 to 24 h, respectively. Plasma and urine samples were stored at −40°C until determination. Plasma concentrations and the urinary excretion of pyrazine were determined by standard physiological pharmacokinetics using the following parameters: area under the curve (AUC) determined by the trapezoidal rule, elimination half-life ($t_{1/2}$), terminal volume of distribution (V_d), total body clearance (Cl_{tot}) and renal clearance (Cl_{ren}).

RESULTS

High-performance liquid chromatography

The chromatographic conditions were determined using six methylpyrazine derivatives and a silica gel column. The mobile phase solvent mixture was optimized to afford sufficient peak separations with resolution factors (R_s) larger than 1.00 and separation factors (α) larger than 1.05. These values are shown in Fig. 2. Typical chromatograms of authentic test mixtures, such as distilled water, human urine and plasma containing six methylpyrazines, each at a concentration of 5 μg/ml, are illustrated in Figs. 2, 3 and 4, respectively. Peak separations between the six pyrazines were sufficient to determine all components simultaneously and quantitatively.

Recovery and calibration

Analytical recoveries of methylpyrazines from plasma and urine, determined by peak-area comparisons between biofluids and authentic solutions in *n*-hexane, are shown in Tables I and II. Recoveries of methylpyrazines from the biofluids were almost quantitative. The method was sufficiently precise, as indicated by the

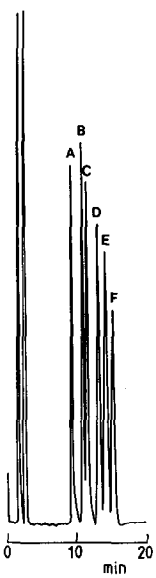

Fig. 2. Typical chromatogram of methylpyrazines. Peaks: A = monomethylpyrazine; B = 2,6-dimethylpyrazine; C = 2,5-dimethylpyrazine; D = 2,3-dimethylpyrazine; E = trimethylpyrazine; F = tetramethylpyrazine. Resolution factors: between A and B, 1.39; B and C, 1.05; C and D, 1.34; D and E, 1.17; E and F, 1.19. Separation factors: between A and B, 1.21; B and C, 1.08; C and D, 1.15; D and E, 1.08; E and F, 1.08.

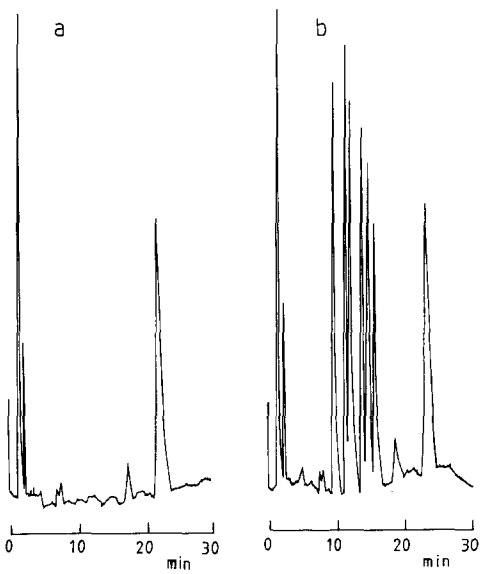

Fig. 3. Typical chromatograms showing (a) methylpyrazine-free human urine and (b) human urine spiked with six methylpyrazines, each at a concentration of 5 μg/ml.

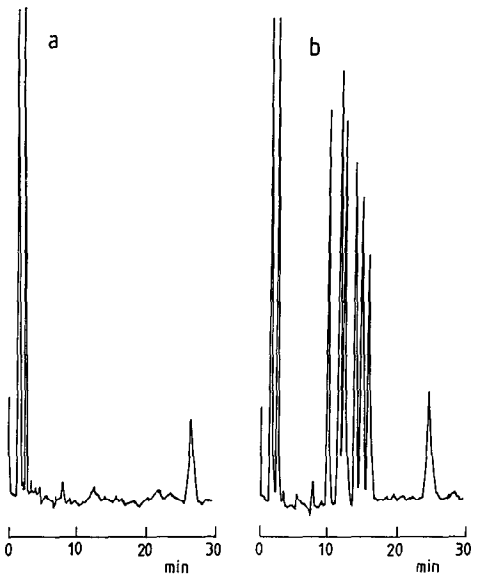

Fig. 4. Typical chromatograms showing (a) methylpyrazine-free human plasma and (b) human plasma spiked with six methylpyrazines each at a concentration of 5 μg/ml.

coefficient of variation, which was less than 4%. Slight peak widening was observed with the syringe-type minicolumn. However, this did not affect either the peak-area or peak-height calibrations. Day-to-day variations could be corrected by the use of authentic biofluids containing known amounts of pyrazines, which were stable for one month at −40°C. These biofluids did not show any decomposition and gave the same peak areas and peak heights after storage as observed immediately after preparation.

The results obtained with biofluids were re-examined using a standard solution in distiled water. There was no significant difference in methylpyrazine recoveries from the distilled water or biofluids. Further experiments to determine cali-

TABLE I

RECOVERIES OF METHYLPYRAZINES FROM HUMAN URINE AT 5 μg/ml (n = 5)

Substitution	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)
Monomethyl	99.0 ± 2.2	2.24
2,3-Dimethyl	97.0 ± 3.1	3.17
2,5-Dimethyl	99.2 ± 1.8	1.85
2,6-Dimethyl	97.6 ± 3.5	3.56
Trimethyl	97.9 ± 2.3	2.37
Tetramethyl	97.7 ± 3.6	3.66

TABLE II

RECOVERIES OF METHYLPYRAZINES FROM HUMAN PLASMA AT 5 μg/ml (n = 5)

Substitution	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)
Monomethyl	98.0 ± 2.8	2.89
2,3-Dimethyl	95.7 ± 1.5	1.51
2,5-Dimethyl	98.5 ± 2.4	2.47
2,6-Dimethyl	96.0 ± 1.6	1.62
Trimethyl	95.7 ± 2.3	2.45
Tetramethyl	94.7 ± 2.6	2.79

bration graphs for methylpyrazines were carried out using standard solutions in distilled water at various concentrations. A linear relationship between peak height and concentration was confirmed for six methylpyrazines (Table III). The graphs were linear from 50 ng/ml to 10 μg/ml. The minimum and maximum concentrations corresponded to injection amounts of 0.5 and 100 ng, respectively. The regression equations, correlation coefficients and statistically significant p values are shown in Table III. The coefficients of variation for six derivatives were all less than 5% over the concentration range studied. The limit of detection was as low as 0.3 ng (30 ng/ml) for each compound at a signal-to-noise ratio of 3.

Pharmacokinetics of 2,5-dimethylpyrazine

The time courses of plasma concentrations of 2,5-dimethylpyrazine after administration in three rats are shown in Fig. 5; urinary excretion profiles are illustrated in Fig. 6. The pharmacokinetic parameters obtained from these data are shown in Table IV. The total excretion of the pyrazine in urine over 24 h was 0.50

TABLE III

CALIBRATION GRAPHS FOR PEAK-HEIGHT DETERMINATION OF METHYLPYRAZINES

Substitution	Regression equation[a]	Correlation coefficient	p Value
Monomethyl	$y = 5490.5x + 52.2$	0.9999	<0.001
2,3-Dimethyl	$y = 4950.1x + 68.9$	0.9999	<0.001
2,5-Dimethyl	$y = 5170.2x + 68.6$	0.9999	<0.001
2,6-Dimethyl	$y = 5793.9x + 64.5$	0.9999	<0.001
Trimethyl	$y = 4119.3x + 112.4$	0.9999	<0.001
Tetramethyl	$y = 3237.2x + 43.8$	0.9999	<0.001

[a] y is peak height observed with the integrator (μV) and x is the concentration of methylpyrazines (μg/ml).

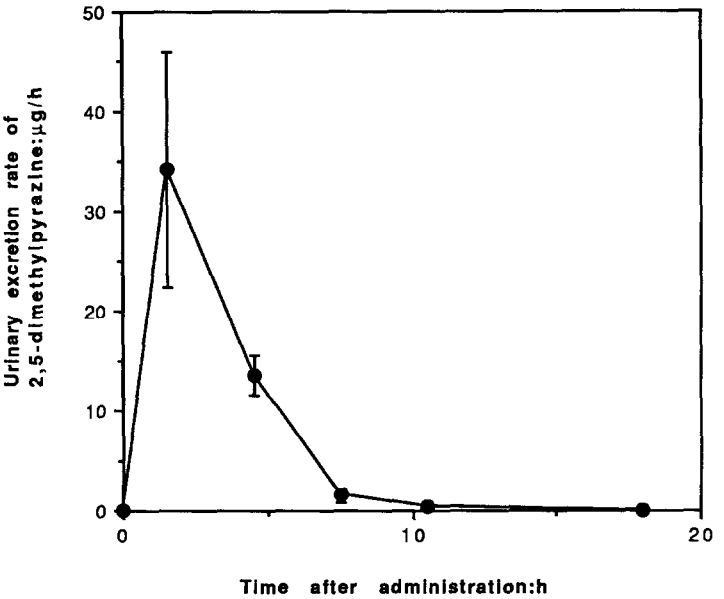

Fig. 5. Time course of 2,5-dimethylpyrazine plasma concentration after intraperitoneal administration to rats (100 mg/kg).

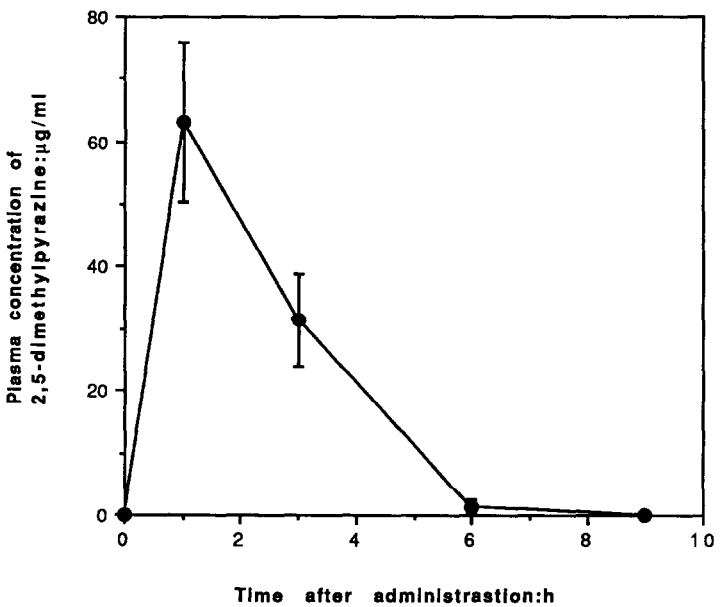

Fig. 6. Time course of 2,5-dimethylpyrazine urinary excretion rates after intraperitoneal administration to rats (100 mg/kg)

TABLE IV

PHARMACOKINETIC PARAMETERS OF 2,5-DIMETHYLPYRAZINE IN RATS

The dose of 2,5-dimethylpyrazine was 100 mg/kg of body weight. For abbreviations, see text.

Parameter	Value (mean ± S.D.)
AUC (μg · h/ml)	168.3 ± 11.2
Cl_{tot} (ml/h)	178.8 ± 11.7
$t_{1/2}$ (h)	0.68 ± 0.02
V_d (ml)	165.8 ± 15.2
Cl_{ren} (ml/h)	0.89 ± 0.26
Total amount of urinary excretion (μg)	148.9 ± 32.9

± 0.11% of the amount administered. This indicated that greater than 99% of the initial dose may have been oxidized to carboxylic acid derivatives [20]. From these data it is apparent that the intraperitoneal absorption of 2,5-dimethylpyrazine into the systemic circulation is rapid, and hepatic metabolism rather than renal clearance may contribute to total body clearance.

DISCUSSION

Head-space techniques which have been applied to determining methylpyrazine derivatives have not given accurate quantitative determinations of aqueous solutions such as rodent and human urine [2,9–12,14]. In most published studies, quantitative analysis was only achieved with head-space gas analysis using GC–MS techniques. Until now, no alternative technique has been used for such a volatile component in an aqueous matrix. The technique described in this paper can be used to analyse aqueous solutions directly, instead of analysing the head-space gas containing only the vaporized fraction of methylpyrazines. To eliminate evaporation or condensation, the organic solvent extracts were handled by a sophisticated syringe-type minicolumn [18,19]. With this technique, the loss of methylpyrazines was minimized, which renders this method applicable to the analysis of biological samples.

The method has been applied to the determination of physiological amounts of methylpyrazines in as much as 10 μl of human urine. However, no apparent peaks were observed on the chromatograms, among a total 180 subjects except in a few cases of urine from women at various stages of pregnancy. As the detection limit of methylpyrazines was as low as 30 ng/ml (0.3 ng of the amount injected), it may be difficult to determine physiological concentrations of methyl pyrazines in human urine. According to known procedures using GC–MS analysis of condensed head-space gas, μg/ml concentrations may be expected [2]. However, the results showed that the concentration of methylpyrazines in human urine may be

less than 30 ng/ml, which corresponds to the detection limit of this method. The pharmacokinetics of 2,5-dimethylpyrazine was studied as a model compound in rats. Some methylpyrazines are used as anticoagulants such as tetramethylpyrazine for congestive heart failure [4] and 2,5-dimethylpyrazine as an antioestrogenic agent [12,13].

The plasma concentration and urinary excretion of 2,5-dimethylpyrazine were sufficiently determined using the minicolumn extraction–injection method. A 10-μl volume of plasma taken from the tail veins of rats at various times after administration was sufficient for the determination. As the amount of sample was as small as possible, it was not necessary to sacrifice animals during timed periods. In this experiment, sampling from rat tail veins was carried out nine times taking the blood into capillary tubes for direct centrifugation before analysis.

Pharmacokinetic profiles obtained by calculation showed clearly that pyrazine could be absorbed immediately after intraperitoneal administration and was eliminated within 10 h following administration (Figs. 5 and 6) with a half-life of less than 1 h (Table IV). However, the recovery of the pyrazine from urine was less than 1% on this dose basis. This means that the predominant fraction of the pyrazine in the systemic circulation should be eliminated through chemical transformation which may be further explained by the significant difference between total and renal clearances shown in Table IV.

The data of this pharmacokinetic analysis are accurate enough to predict the systemic behaviour of the agent which may contribute to pharmacological action in animals.

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