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Journal of Chromatography B, 726 (1999) 195–201

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic method for simultaneous determination of [1-methyl-^{14}C]caffeine and its eight major metabolites in rat urine

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Received 7 October 1998; received in revised form 15 January 1999; accepted 15 January 1999

Abstract

A selective and sensitive reversed-phase liquid chromatographic method was developed for the simultaneous analysis of [1-Me-^{14}C]caffeine and its eight major radiolabelled metabolites in rat urine. The separation of the complex mixture of caffeine metabolites was achieved by gradient elution with a dual solvent system using an endcapped C_{18} reversed-phase column, which in contrast to commonly used C_{18} reversed-phase columns also allows the separation of the two isomers of 6-amino-5-(*N*-formylmethylamino)-1,3-dimethyluracil (1,3,7-DAU), a caffeine metabolite of quantitative importance predominantly occurring in rat. As caffeine is metabolised primarily by members of the cytochrome P450 1A (CYP1A) subfamiliy, determination of the pattern of caffeine metabolites in rat urine enables analysis of activities of this important enzyme subfamily in vivo. Since CYP1A is suggested to be involved in the detoxification of bilirubin, the assay may be applied to search for untoxic inducers of CYP1A which might be of pharmacological interest in the treatment of hyperbilirubinaemia.

Keywords: Caffeine; Cytochromes

1. Introduction

Cytochrome P450 1A (CYP1A) isoformes, cytochromes P450 inducible by polycyclic aromatic hydrocarbons, are of toxicological and pharmacological importance because of their key role in toxification and detoxification of xenobiotics and in chemical carcinogenesis [1,2]. Furthermore, various studies suggest that CYP1A isoforms participate in the hydroxylation of bilirubin, a neurotoxic product of hemoglobin catabolism, thereby facilitating bilirubin elimination [3–7]. Therefore, induction of CYP1A enzymes might be a therapeutical approach for the treatment of congenital hyperbilirubinaemia [5]. Nevertheless, the search for new and safe inducers of CYP1A requires the use of animal models, e.g. the Gunn rat, which in parallel to hyperbilirubinaemia in humans also exhibits elevated

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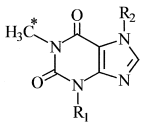
0378-4347/99/$ – see front matter
PII: S0378-4347(99)00040-7

plasma bilirubin levels due to a genetic defect in bilirubin detoxification [8].

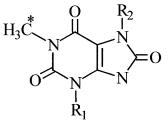
In the past years, caffeine has been shown to be a useful enzyme probe for assessing the activity of CYP1A isoforms in humans. Differences in enzyme activity of CYP1A cause alterations in the quantitative rate of formation of certain caffeine metabolites, resulting in changes of the pattern of metabolites in urine [9]. Furthermore, an increase in the activity of CYP1A proteins leads to an accelerated elimination of caffeine in humans. Therefore, caffeine clearance has also been established as a measure of CYP1A activity in humans [10]. In contrast to the well characterized conditions in humans, little is known about the assessment of the CYP1A activity in rats on the basis of their caffeine metabolism. While most of the steps of biotransformation in metabolism of caffeine in humans are mediated at least in part by the CYP1A isoforms [9,11,13], further enzymes, e.g. flavin-containing monooxygenases are involved in caffeine metabolism in rats [11,12]. Thus, due to involvement of other enzymes in caffeine metabolism, caffeine clearance alone may not represent a concise measure for CYP1A activity in rats.

Caffeine (1,3,7-trimethylxanthine) in humans as well as in rats is extensively metabolised to various methylxanthines, methylurates and uracil derivatives (for chemical structures see Fig. 1). The initial metabolism of caffeine leads to 1-, 3- and 7-demethylations (yielding theobromine, paraxanthine, and theophylline, respectively) and to 8-hydroxylation to 1,3,7-trimethyluric acid [14]. Characteristic of caffeine biotransformation in rats is the formation of 6-amino-5-(*N*-formylmethylamino)-1,3-dimethyluracil (1,3,7-DAU), a metabolite with an opened imidazole ring, in amounts of quantitative importance [15–17].

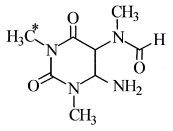
In further metabolism the three dimethylxanthines may be demethylated to form 1-, 3- or 7-methylxanthines or, alternatively, 8-hydroxylated to generate the corresponding dimethyluric acids. Eventually, 8-hydroxylation of the monomethylxanthines leads to the formation of the corresponding monomethyluric acids [14]. Furthermore, allantoin and urea derivatives have been described as metabolites of caffeine in rats [15]. The formation of paraxanthine is predominant in comparison to the other demethylations [18,19].



R_1	R_2	Metabolite
CH_3	CH_3	caffeine (1,3,7-X)
CH_3	H	theophylline (1,3-X)
H	CH_3	paraxanthine (1,7-X)
H	H	1-methylxanthine (1-X)



R_1	R_2	Metabolite
CH_3	CH_3	1,3,7-trimethyluric acid (1,3,7-U)
CH_3	H	1,3-dimethyluric acid (1,3-U)
H	CH_3	1,7-dimethyluric acid (1,7-U)
H	H	1-methyluric acid (1-U)



6-amino-5-(N-formylmethylamino)-1,3-dimethyluracil (1,3,7-DAU)

Fig. 1. Radiodetectable metabolites of [1-Me-^{14}C]caffeine.

Several HPLC methods to determine caffeine and its metabolites in human urine and plasma have been reported [20–24]. However, due to the more complex metabolism in rats, these methods are not feasible for analysis of the caffeine metabolite spectrum in rats. Furthermore, most of the assays for the analysis of caffeine metabolism in humans are

restricted to the determination of distinct caffeine metabolites, e.g. the dimethylxanthines, or to the analysis of plasma. Therefore, we were interested in establishing a selective HPLC assay for the simultaneous determination of [1-Me-^{14}C]caffeine and its major radiolabelled metabolites in rat urine which might be suitable for the investigation of CYP1A activities in rats. [1-Me-^{14}C]caffeine was chosen as a substrate in our investigations in order to analyse a broad spectrum of caffeine metabolites (like di- and monomethylxanthines, uric acids and 1,3,7-DAU) without interference with the urine matrix. Since 3-demethylation of caffeine is the major metabolic pathway in caffeine metabolism in rats [16], [1-Me-^{14}C]caffeine allows the determination of the major caffeine metabolites with the exception of 1-demethylated products.

2. Experimental

2.1. Reagents

Caffeine, 1,3,7-trimethyluric acid, 1-methylxanthine, 1-methyluric acid, 1,3-dimethylxanthine, 1,3-dimethyluric acid, 1,7-dimethylxanthine and 1,7-dimethyluric acid were obtained from Sigma (St. Louis, USA). [1-Me-^{14}C]caffeine (specific activity 2.2 GBq/mmol) was purchased from Du Pont NEN (Bad Homburg, Germany). 6-Amino-5-(*N*-formylmethylamino)-1,3-dimethyluracil was a gift from Nestlé Research Center (Lausanne, Switzerland). HPLC grade methanol and dimethylformamide were obtained from Merck (Darmstadt, Germany). Other chemicals were of p.a. purity.

2.2. Instrumentation and HPLC conditions

The HPLC system consisted of a Kontron Instruments pump system (Ecking, Germany) coupled to a six-port injector which was equipped with a 100 μl fixed-volume sample loop (Rheodyne, Cotati, CA, USA). A Kontron Instruments D332 UV-detector (Ecking, Germany) and a Berthold LB 506 radiodetector fitted with a 400 μl flow cell were used in series (Wildbad, Germany). The sample wavelength was set to 280 nm. Data acquisition and transformation were accomplished by the JMBS Developments winflow data system (Le Fontanil, France). The analytical column was a Merck LiChrosphere 100 RP-18 endcapped 5 μm (Darmstadt, Germany).

A dual solvent system was used as the mobile phase. The eluents were of the following compositions. Eluent A: 10 m*M* sodium acetate buffer (adjusted to pH 5.5 with acetic acid) methanol dimethylformamide (99.0:0.5:0.5). Eluent B: methanol–eluent A (50:50). The flow-rate was 1.5 ml/min. Separation was performed at ambient temperature using the following linear gradient segments: 0% B at 0 min to 10% B at 10 min, 10% B at 10 min to 60% B at 35 min.

2.3. Animal experiments

Male Gunn rats obtained from Harlan-Winkelmann (Zeist, The Netherlands) were fed an Altromin® standard diet (Altromin, Laage, Germany) and water ad libitum. For induction of CYP1A the rats were treated with 80 mg 5,6-benzoflavone/kg body weight in 4 ml/kg corn oil, injected intraperitoneally (i.p.) 48 h before application of caffeine. In control experiments the animals recieved 4 ml/kg corn oil i.p.. Caffeine was administered as a mixture of 740 kBq/kg [1-Me-^{14}C]caffeine and 10 mg/kg non-radiolabelled caffeine in 8 ml/kg sodium chloride (0.9%) i.p.. Animals were kept in metabolic cages and urine was collected over 24 h in fractions of 3 h during the first 12 h. The urine was frozen and stored at −20°C. Additionally, faeces were collected and exhaled $^{14}CO_2$ was trapped in a solution of 12% ethanolamine in ethanol over 24 h.

2.4. Urine sample treatment

For sample preparation the urine was sonicated for 3 min and the fractions from 0–12 h were pooled. From the available urine volume a tenth was transferred into a test-tube and 20 μl acetic acid (100%) were added to reach a pH below 5. No extraction step was required before urine samples were chromatographed. To clear the urine from insoluble particles it was centrifuged for 5 min at 15 000 *g*. Subsequently, a 100 μl aliquot was injected into the chromatograph.

3. Results and discussion

3.1. Sample preparation and separation

The aim of this analytical assay was the simultaneous determination of caffeine and eight of its major metabolites such as 1-methyluric acid, 1-methylxanthine, 1,3-dimethyluric acid, theophylline, 1,7-dimethyluric acid, paraxanthine, 1,3,7-trimethyluric acid, and 6-amino-5-(*N*-formylmethylamino)-1,3-dimethyluracil (1,3,7-DAU) in rat urine. The analysed compounds are summarised in Fig. 1 with their chemical structures and the abbreviations. To ensure that all 9 compounds could be analysed simultaneously without interference with the complex urine matrix, radiolabelled caffeine ([1-Me-^{14}C]caffeine) was used as a substrate in the investigations.

The simultaneous determination of nine substances may complicate sample preparation, particularly when substances with differences in their polarity like ureates and xanthines are to be analyzed. For this assay liquid–liquid extraction and liquid–solid extraction were tested, but in our experience, neither of both allowed a recovery which was greater than 70% for all metabolites (data not shown). Thus, a sample preparation procedure without an extraction step was developed, which only consists of pH adjustment and centrifugation of the urine sample. This leads to a shorter sample preparation time and allows a quantification of the radiolabelled metabolites based only on the measured radioactivity in the corresponding peak without consideration of any extraction efficiency. A peculiarity of the caffeine biotransformation in rat in contrast to human metabolism is the formation of 1,3,7-DAU in considerable amounts, an uracil derivative, which exists as two isomers [15]. In liquid chromatography these isomers are separated into two peaks (Fig. 2) leading to a more complex pattern of peaks. Analysis of the structure of 1,3,7-DAU in aqueous solution by nuclear magnetic resonance suggested that the two peaks could be assigned to two rotamers with differences in the stereochemistry of the formamide structure (data not shown).

When HPLC is performed with a C_{18} column which is commonly used in analysing caffeine and metabolites, the two 1,3,7-DAU rotamers interfere with discrimination of other caffeine metabolites in that peaks overlap [15]. However, a C_{18} endcapped column allows the complete separation of both isomers of 1,3,7-DAU and of all labelled methylxanthines and methyluric acids. This is demonstrated in Fig. 3A, showing the separation of a standard cocktail containing non-radiolabelled caffeine and its eight metabolites in concentrations of 10 μg/ml (100 μg/ml 1,3,7-DAU, respectively) in acetate buffer. Fig. 3B shows the separation of a urine sample obtained from a Gunn rat, which was treated with [1-Me-^{14}C]caffeine at a dosage of 740 kBq/kg. Radiolabelled metabolites, formed by in vivo metabolism of [1-Me-^{14}C]caffeine were detected in the urine sample in different amounts. As apparent from Fig. 3B, the applied HPLC conditions allowed the separation of the major radiolabelled methylxanthines, methyluric acids and uracil derivatives as well as other non-identified metabolites, which were eluated with shorter retention times. Peaks in radiochromatograms were identified according to their retention times which were compared to retention times of the unlabelled standard metabolites obtained in a separation of a standard cocktail. Fig. 3A and B demonstrate that both separations were comparable in their peak pattern which supports the validity of identification of metabolites in analysis of rat urine. Analogous to the analysis of the non-radiolabelled metabolites (Figs. 2 and 3A), separation of radioactive 1,3,7-DAU in the urinary sample also led to the

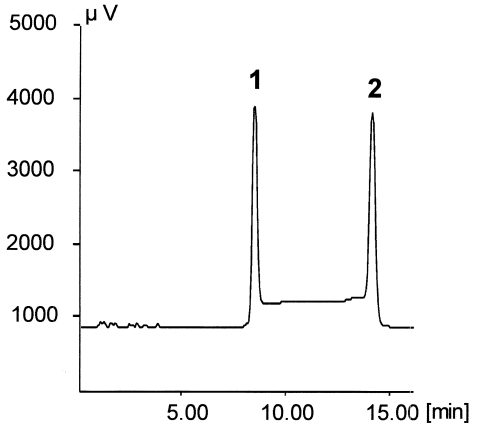


Fig. 2. UV chromatogram of non-radiolabelled 1,3,7-DAU in a concentration of 100 μg/ml in acetate buffer. Peaks: 1=first isomer of 1,3,7-DAU, 2=second isomer of 1,3,7-DAU.

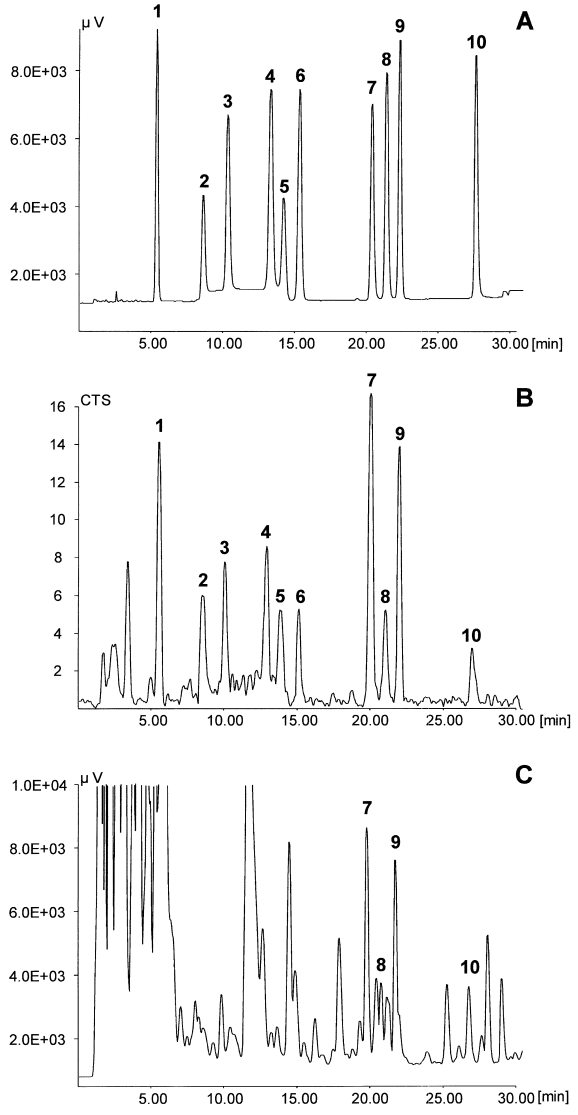


Fig. 3. (A) UV chromatogram of non-radiolabelled caffeine and its eight selected metabolites as references in acetate buffer (in concentrations of 100 μg/ml for 1,3,7-DAU and 10 μg/ml for the other metabolites, respectively). Peaks: 1=1−U, 2=1,3,7−DAU (first isomer), 3=1−X, 4=1,3−U, 5=1,3,7−DAU (second isomer), 6=1,7−U, 7=1,7−X, 8=1,3−X, 9=1,3,7−U, 10=1,3,7−X (for abbreviations and chemical structures see Fig. 1). (B) Radio chromatogram of a urine sample containing [1-Me-^{14}C]caffeine and radiolabelled metabolites obtained from a Gunn rat. Peak specification by numbers is the same as in panel A. (C) UV chromatogram of the same urine sample as analysed in Fig. 3B.

formation of a plateau between the two peaks of its isomers. Since the peaks 3 and 4 corresponding to 1-X and 1,3-U, respectively, are eluted within this area (Fig. 3B) it appears feasible to subtract the plateau as background radioactivity.

Fig. 3C demonstrates a UV-chromatogram of the same urine sample as analysed in Fig. 3B. While caffeine and some of its metabolites such as 1,3-X, 1,7-X and 1,3,7-U can be also determined according to the UV chromatogram of the urine sample, the simultaneous determination of all eight metabolites (plus caffeine) is not feasible without the use of radiolabelled caffeine as a substrate, due to interference with the urine matrix.

Precision of the retention times (intra- and inter-day) was determined by analysing a urine sample containing radiolabelled caffeine and metabolites five times over the same day and over different days. The results are shown in Table 1. The values of the coefficients of variation (C.V.) ranging between 0.8 and 5.5% demonstrate that the separation is highly consistent.

Owing to the lack of available [1-Me-^{14}C]-labelled standards no accuracy of the assay could be determined. For estimating the precision of quantitative determination by the method, a urine sample representing the metabolic pattern of a Gunn rat treated with [1-Me-^{14}C]caffeine in a dosage of 740 kBq/kg body weight was used. The sample contained all detectable metabolites in different amounts. Separation was performed five times over one, and five times over five consecutive days, respectively. The concentrations of the metabolites were quantified according to the determined radioactivity of the corresponding peak and expressed as pmol/ml. Data on repeated analysis of the urine sample are presented in Table 2.

In view of the fact that nine substances, one of which is divided into two peaks, can be detected and quantified simultaneously, good values for coefficients of variation were obtained ranging between 0.5 and 8.4%.

The lower limit of detection was estimated as five times the noise value which is less than 1 pmol/ml. All [1-Me-^{14}C]-labelled metabolites are expected to exhibit a similar limit of detection since all metabolites have the same specific activity. In the mean of five experiments 72.3±4.9% of the administered

Table 1
Inter- and intra-day precision of retention times

Metabolite[a]	Intra-day		Inter-day	
	Retention time (mean±S.D., n=5) (min)	C.V.[b] (%)	Retention time (mean±S.D., n=5) (min)	C.V.[b] (%)
1-U	5.5±0.1	1.5	5.5±0.3	4.5
1,3,7-DAU	8.8±0.2	2.1	8.7±0.4	4.4
(first isomer)				
1-X	10.3±0.2	1.5	10.0±0.2	2.1
1,3-U	13.0±0.2	1.5	12.8±0.7	5.5
1,3,7-DAU	14.3±0.5	3.4	14.0±0.5	3.9
(second isomer)				
1,7-U	14.8±0.2	1.4	14.7±0.6	4.4
1,7-X	20.3±0.2	1.0	20.0±0.5	2.4
1,3-X	21.3±0.2	0.8	21.0±0.5	2.4
1,3,7-U	22.1±0.2	0.9	21.8±0.5	2.4
1,3,7-X	27.7±0.2	0.8	27.1±0.5	1.8

[a] For abbreviations of the metabolites see Fig. 1.
[b] Coefficient of variation.

dose of radioactivity was excreted into the urine within the first 24 h. The determined caffeine and its metabolites accounted for more than 70% of total radioactivity in the urine.

3.4. Application of the method

Cytochrome P450 enzymes of the CYP1A subfamily which are of major importance for the biotransformation of innumerable xenobiotic compounds have been also suggested to be involved in the detoxifaction of bilirubin. CYP1A is inducible by polycyclic aromatic hydrocarbons like 5,6-benzoflavone (BNF) [25] and thus exhibits great changes in its activity. Therefore, a method to determine CYP1A activity in vivo is of great pharmacological and toxicological interest. In the past years, caffeine has been shown to be a useful probe to assess the activity of CYP1A in humans, however, no assay has been described which allows determination of

Table 2
Precision of quantitative determination of [1-Me-^{14}C]caffeine and its major metabolites in rat urine

Metabolite[a]	Intra-day		Inter-day	
	Amount[b] (mean±S.D., n=5) (pmol/ml)	C.V.[c] (%)	Amount[b] (mean±S.D., n=5) (pmol/ml)	C.V.[c] (%)
1-U	173.3±4.0	2.3	174.4±6.3	3.6
1,3,7-DAU	183.2±9.7	5.3	186.5±5.72	3.1
1-X	122.5±2.2	1.8	127.5±4.9	3.8
1,3-U	157.8±2.3	1.5	149.5±9.1	6.1
1,7-U	69.1±4.7	6.9	72.1±3.2	4.4
1,7-X	269.1±7.2	2.7	274.3±13.6	5.0
1,3-X	76.9±1.6	2.1	84.9±7.1	8.4
1,3,7-U	160.1±10.5	6.6	167.7±0.8	0.5
1,3,7-X	59.1±3.8	6.4	61.8±1.8	2.9

[a] For abbrevations of the metabolites see Fig. 1.
[b] Calculated from peak radioactivity in reference to [1-Me-^{14}C]caffeine standard.
[c] Coefficient of variation.

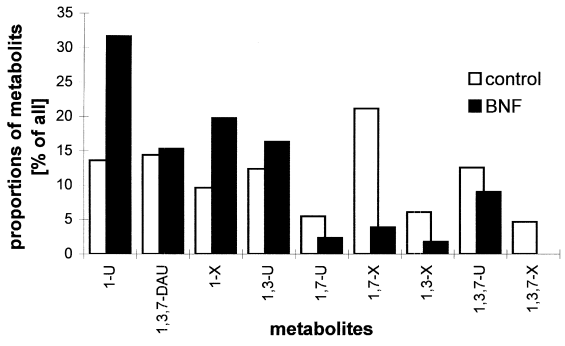


Fig. 4. Proportions of caffeine and its metabolites in Gunn rat before and after treatment of the rat with 5,6-benzoflavone (BNF) for CYP1A induction. For abbreviations and chemical structures see Fig. 1.

CYP1A activity in rats in vivo. On the other hand, the search for new inducers of CYP1A as possible therapeutics in the treatment of hyperbilirubinaemia requires the use of animal models, e.g. the Gunn rat which also exhibits elevated bilirubin levels due to insufficient bilirubin elimination. Thus, this HPLC method was applied to analyse caffeine and its eight major 1-methylated metabolites in urine samples from Gunn rats treated with the CYP1A inducer BNF. The results shown in Fig. 4 demonstrate the pattern of caffeine metabolites obtained in urine from a rat before (white bars) and after induction (black bars) of CYP1A with BNF. As apparent, treatment with BNF markedly increased the formation of 1-U and 1-X, whereas the proportion of 1,7-X decreased. Therefore, the proportion of these metabolites or the ratio of the proportions of (1-U+1-X)/1,7-X is an excellent indicator of CYP1A activity. Similar results as observed in caffeine metabolism in Gunn rats were obtained in experiments with Wistar rats (data not shown) which demonstrates that the described assay is not limited to measurement of CYP1A activity in Gunn rats.

In conclusion, these data demonstrate that the described HPLC method is useful for analysing [1-Me-^{14}C]caffeine and its eight major radiolabelled metabolites in rat urine and offers the possibility of in vivo-assessment of CYP1A activities in the rat.

Acknowledgements

This study was supported by the Lower Saxony–Israel cooperative research program of the Volkswagen foundation. We are also grateful to Dr. Philippossian, Nestlé Research Center (Lausanne, Switzerland) for providing 1,3,7−DAU as a reference substance.

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