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Journal of Chromatography A, 870 (2000) 463–467

JOURNAL OF CHROMATOGRAPHY A

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Determination of apigenin in rat plasma by high-performance liquid chromatography

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Abstract

Apigenin (4′,5,7-trihydroxyflavone, AP) belongs to a less-toxic and non-mutagenic flavone subclass of flavonoids, the biotransformation and metabolism of which have been little studied until now. Therefore, this study is focussed on the determination of AP in free form. AP was administered to rats via the i.p. route (25 mg kg^{-1}) and then the blood was collected at 10, 15, 30 and 45 min after injection. Methanol was used for rat plasma deproteinization. The HPLC assay (mobile phase, 2% formic acid–acetonitrile–methanol, 40:35:25, v/v; flow-rate, 1 ml min^{-1}; UV detection at 349 nm) for AP determination was validated and used for the quantification of AP in rat plasma. The unknown concentration was calculated from the equation obtained by the least-squares regression analysis ($y = 0.521x + 1.130$, $r^2 = 0.998$). The highest concentration of AP in plasma was found to be 30 min after injection. The concentration profile of AP obtained here may contribute to until known results about AP metabolism. They could be applied to other studies of AP or related flavonoids because of favourable effects on human health.

Keywords: Apigenin; Flavonoids

1. Introduction

Flavonoids are naturally occurring polyphenolic compounds. Much attention is now being focussed on flavonoids because of their pleiotropic effects [1–3]. Firstly, Szent-Györgyi referred to their ability to decrease capillary permeability and fragility [4]. At the present time, the preferred study of the flavonoids is aimed at their antioxidative properties which are in relation to their free-radical scavenging, metal-chelating ability and inhibitory effect on lipid peroxidation [5–8]. Flavonoids have been also reported to exert an anti-inflammatory [9,10], anti-allergic [11,12] and antidiabetic effects [13].

A number of epidemiological studies show a protective effect of vegetables and fruits against cancer [14,15]. The inhibition of processes during carcinogenesis is suggested to be due to multiple biological effects of flavonoids [16].

Apigenin (AP, Fig. 1), a less-toxic and non-mutagenic flavone subclass of flavonoids, is one of the most investigated flavonoids with promising chemopreventive activity against skin cancer. AP suppressed 12-*O*-tetradecanoylphorbol-13-acetate-mediated tumour promotion of mouse skin [17–19].

The metabolism and the pharmacokinetic data of flavonoids in biological fluids were until recently little studied [3]. The biotransformation of AP has

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)00939-5

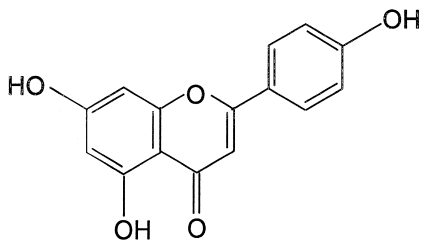

Fig. 1. Chemical structure of apigenin.

been investigated in rat liver microsomes in vitro [20]. Its levels have been determined in human urine [21] and in epidermal extracts from mice, both by HPLC methods [17]. Several HPLC methods have been developed for detection of various flavonoids in plant materials [22–24] and/or in biological samples (fluids, tissue) [25–27], but with lack of specificity and sensitivity to AP.

In this study, we developed a rapid and sensitive HPLC method for the detection of AP in rat plasma. The isocratic HPLC method with UV detection was used for the determination of concentration profile of unmodified (free form) AP in rat plasma after i.p. administration (25 mg kg^{-1}).

2. Experimental

2.1. Chemicals

AP (4′,5,7-trihydroxyflavone) and acetonitrile were purchased from Fluka (Buchs, Switzerland). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). Acetonitrile and methanol were of HPLC grade and all other chemicals used were of analytical grade.

2.2. Standards

AP was dissolved in dimethylsulfoxide at the concentration of 10 mmol l^{-1} as stock solution and stored at −20°C. Standard samples for calibration were prepared by dilution of stock solution giving final concentration of AP in the range of concentration from 0.5 to 200 μmol l^{-1}.

2.3. Animals

Male (300–350 g) and female (200–250 g) Sprague–Dawley rats were housed in experimental groups (five animals) according to the guidelines for care and use of laboratory animals. The experiments were approved by the Ethical Committee of the Cancer Research Institute. The animal facility of the Cancer Research Institute SAS (Bratislava, Slovak Republic) is certified for performing scientific research on animals.

2.4. Plasma samples

Rats received a bolus dose of AP of 25 mg kg^{-1} via the i.p. route. The blood was collected from the heart by heparinized syringe at 0, 10, 15, 30 and 45 min after administration, centrifuged at 8000 *g* for 5 min at 4°C. Five rats were used in each time point. The resulting plasma was collected, frozen and kept at −70°C until analysis.

2.5. Treatment of plasma samples

The plasma (0.5 ml) was deproteinized by adding 0.8 ml of methanol, vortex-mixed for 60 s and centrifuged at 2200 *g* for 15 min at 4°C. The supernatant (1.2 ml) was collected into the tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 250 μl of methanol and chromatographically analyzed.

2.6. Chromatographic conditions

The HPLC system consisted of Rheodyne 7010-091 injector (20-μl loop), 2150 HPLC pump coupled with a 2152 HPLC controller (LKB Bromma, Sweden), variable UV–Vis 970 intelligent detector (Jasco, Tokyo, Japan) set at 349 nm. Data were evaluated by software CSW (Data Appex, Prague, Czech Republic). AP was separated on Separon (Tessek, Prague, Czech Republic) SGX C_{18} column (150×3 mm I.D., 7 μm particle size). The mobile phase, consisting of 2% formic acid–acetonitrile–methanol (40:35:25, v/v), was run at a flow-rate of 1 ml min^{-1} at ambient temperature.

2.7. Validation

Calibration of the chromatogram was accomplished using the external standard method. Linear calibration curves were performed from the peak heights of AP to the external standard AP by spiking drug-free rat plasma with standard solutions of AP to give a concentration range of 0.5–200 μmol l^{-1}. The samples were taken through the deproteinization procedure described above and the peak height plotted against the corresponding concentration.

The unknown AP concentrations in plasma samples were determined from the equation generated by the least-squares regression analysis.

The recovery of AP in rat plasma was calculated by comparing peak heights for plasma samples spiked with AP in five replications with those of standard solutions at five of concentrations of 0.5, 5, 50, 100 and 200 μmol l^{-1}. Inter- and intra-assay precision and accuracy were determined by injecting of standard solutions of 0.5, 5, 50, 100 and 200 μmol l^{-1} on the same day and for 7 days, respectively. Similarly, validation of the assay was performed for AP-spiked rat plasma in five replications. The detection limit for AP was determined at a signal-to-noise ratio of 3:1.

3. Results and discussion

Several assays for the determination of AP and/or related flavones have been described [20,21,28,29]. Based on these HPLC assays, we optimized the chromatographic conditions for the determination of AP in rat plasma. The separation of AP was performed isocratically in reversed-phase mode. To determine the optimal composition of the mobile phase, several mixtures were used. The concentration of formic acid and organic modifier were changed. The best separation has been provided when the mobile phase contained 2% of formic acid–acetonitrile–methanol (40:35:25, v/v). The retention time of AP was 2.45 min and retention factor was 1.31.

The attempt to extract AP using a C_{18} solid-phase extraction (SPE) cartridge failed obtaining low recoveries (below 70%). In the case of urine samples containing AP, SPE was successfully used [21]. In our assay, rat plasma was pre-treated by protein precipitation with methanol. The recoveries of AP from rat plasma were 93.6, 103.6, 95.3, 96.8 and 94.9% for concentrations of 0.5, 5, 50, 100 and 200 μmol l^{-1}, respectively.

Regression analysis was performed on the calibration curve in plasma. The calibration curve obtaining by injection of deproteinized rat plasma spiked with AP and using peak heights over the concentration range of 0.5–200 μmol l^{-1} was linear. No interfered peaks were observed within the time in which AP was detected. The equation for regression line was found to be $y = 0.521x + 1.130$, correlation coefficient 0.998. The detection limit for AP at a signal-to-noise ratio of 3:1 was found to be 0.1 μmol l^{-1}.

The reproducibility of the assay was evaluated by intra- and inter-day variabilities. The retention times of AP showed inter- and intra-day variation less than 1% (RSD) and the peak heights determined varied less than 5% (RSD). The results of assay validation for AP in rat plasma are included in Table 1.

The mean plasma concentrations of free form of AP at 10, 15, 30 and 45 min after treatment of 25 mg kg^{-1} i.p. dose are in Table 2. The chromatograms of blank plasma, spiked plasma and plasma after 10 min treatment are depicted in Fig. 2. The highest concentration of AP in rat males and females was reached 30 min after AP injection. In males, the concentration of AP is relatively kept at the same

Table 1
Validation of assay for AP in rat plasma

Spiked conc. (μmol l^{-1}), added	Mean calculated conc. (μmol l^{-1}), found	RSD (%)
Intra-day		
0.5	0.483±0.026[a]	5.4
5	4.905±0.183	3.7
50	50.184±1.106	2.2
100	100.282±1.097	1.1
200	200.104±1.086	0.5
Inter-day		
0.5	0.481±0.027	5.6
5	4.895±0.191	3.9
50	49.253±1.117	2.3
100	100.145±1.123	1.1
200	200.098±0.840	0.4

[a] Data are expressed as mean ±SD, $n = 5$.

Table 2
The concentration of AP found after 10, 15, 30 and 45 min treatment

Time (min)	Concentration found (μmol l^{-1})	
	Male	Female
10	11.246±2.148[a]	9.896±2.018
15	11.179±2.195	17.218±7.099
30	30.953±11.284	26.218±19.366
45	21.734±5.328	22.700±6.159

[a] Data are expressed as mean ±SD, $n=5$.

level during 15 min and slowly decreased at 45 min. On the other hand, in females, the AP concentration was linearly increased during 30 min and similarly to males, was brought down at 45 min.

Similar results were observed after intestinal absorption of luteolin and luteolin 7-*O*-β-glucoside in rats. The concentration of luteolin and its conjugates in rat plasma increased to the highest level 15 and 30 min after gastric intubation (50 μmol kg^{-1}) and decreased gradually. LC–MS analysis showed that the main metabolite of luteolin in plasma was a monoglucuronide [29].

Nielsen and Dragsted have developed a column-switching HPLC assay for the determination of AP in human urine. There is supposed that potential metabolite of AP is its 4′-methylated derivative, acacetin, in human urine [21]. The main metabolite of AP after in vitro biotransformation by rat liver microsomes, tentatively identified on the base of retention time, was 3′-hydroxylated compound, luteolin [20]. The authors have also reported the presence of AP unmodified in the results described above. It is suggested, that this free form of AP could escape glucuronidation and/or hepatic sulfation/methylation. A previous studies showed that the other flavonoids, e.g., naringenin and diosmetin circulate in the blood whether in glucuronide form or in free form [26,30].

The relation of the number of hydroxyl groups substituted on the B ring to ·OH scavenging activity [31], and relation of dihydroxyl groups at 3′- and 4′-positions to antioxidant activity, and that the antioxidant activity decreases with methylation of the hydroxyl groups [32], have all been reported.

In our experiments, rat plasma after i.p. administration contained only unmodified AP which probably escaped other metabolization. The concentration profile of AP in rat plasma showed its relatively high level in plasma. An unknown peak was observed in

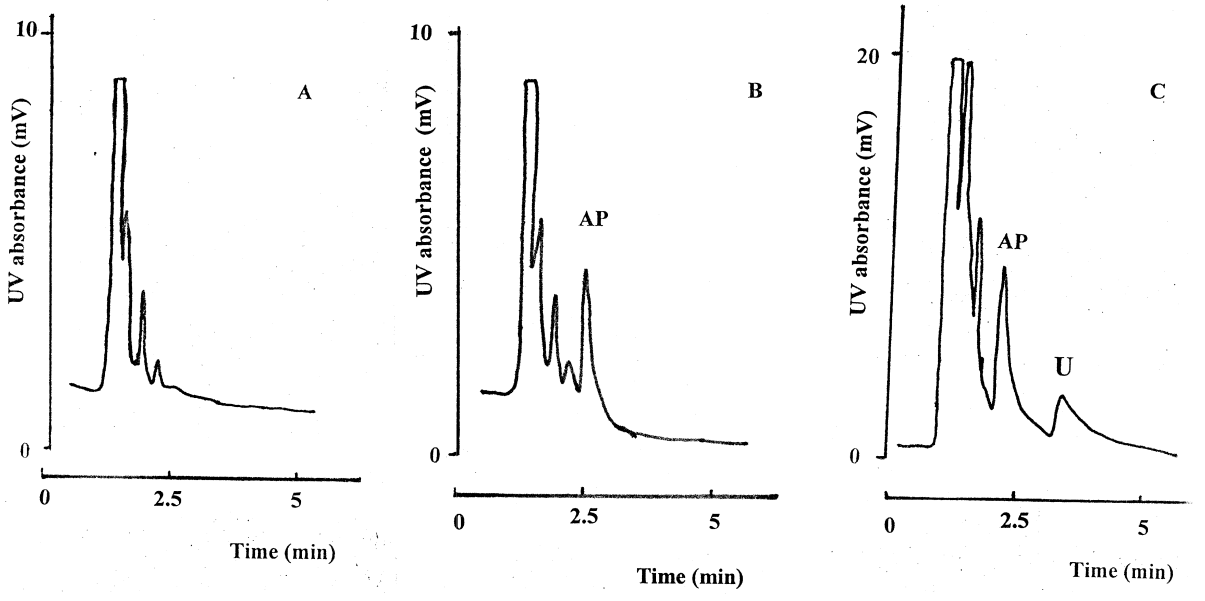

Fig. 2. The chromatograms of extracted blank plasma (A); extracted plasma sample by spiking with 1 μmol l^{-1} of AP (B); extracted plasma sample taken 10 min after i.p. dose of 25 mg kg^{-1} (C). Chromatographic conditions as described in Section 2. AP, apigenin; U, unknown peak.

the chromatogram (see Fig. 2), the concentration of which was unchanged (assumption on the base of its peak height) for the time points studied. No AP was detected in rat plasma 60 and 90 min after injection as well as in rat liver 30 min after i.p. injection (data not shown).

On the basis of our results and other results reported, we can state that biological activity, the metabolism of flavonoids is dependent on their structure (the number of hydroxyl groups and their position). Further, it is important to maintain flavonoid level in blood to achieve any effect on health, but other investigations are needed to clarify their optimal intake, biotransformation, metabolism and final effect.

4. Conclusions

The RP-HPLC method described here has been successfully applied to the determination of AP in rat plasma and to monitoring of AP for four time points after i.p. administration. This method can be adapted for the analysis of similar compounds. Since the distribution, biotransformation and metabolism of AP are not yet elucidated, and it seems promising as a cancer preventive agent, this monitoring could aid to other studies on AP or related flavones.

Acknowledgements

The authors want to thank ChromSpeck Slovakia Ltd., the distributor of analytical instrumentation (UNICAM, Gynkotek), for support to take part in the 23rd International Symposium on High-Performance Liquid Phase Separations and Related Techniques (HPLC'99), where these results were presented.

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