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Development and validation of a liquid chromatographic–tandem mass spectrometric method for the determination of caffeic acid phenethyl ester in rat plasma and urine

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Abstract

Caffeic acid phenethyl ester (CAPE) is one of the most bioactive compounds of propolis, a resinous substance collected and elaborated by honeybees. A new liquid chromatography–electrospray ionisation tandem mass spectrometric method was developed and validated for its determination in rat plasma and urine, using taxifolin as internal standard. After sample preparation by liquid/liquid extraction with ethyl acetate, chromatographic separations were carried out with an ODS-RP column using a binary mobile phase gradient of acetonitrile in water. Detection was performed using a turboionspray source operated in negative ion mode and by multiple reaction monitoring. The method was validated, showing good selectivity, sensitivity (LOD = 1 ng/ml), linearity (5–1000 ng/ml; $r \geq 0.9968$), intra- and inter-batch precision and accuracy ($\leq$14.5%), and recoveries (94–106%) in both plasma and urine. Stability assays have shown that CAPE is rapidly hydrolysed by plasmatic esterases, which are however inhibited by sodium fluoride. The method was applied to the determination of CAPE levels in rat plasma and urine after oral administration, showing that CAPE is rapidly absorbed and excreted in urine both as unmodified molecule and as glucuronide conjugate.

Keywords: Caffeic acid phenethyl ester; Bioavailability

1. Introduction

Propolis, or "bee glue", is a natural sticky dark-coloured product collected by worker bees from the leaf buds of different tree species, enriched with other plant materials and with secreted substances from bee metabolism [1,2]. Usually, raw propolis is composed of 50% resins and balsams, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% various other substances [3], but the proportion of these substances depends on the geographical region as well as on the period of collection [4]. The term "propolis" derives from the Greek *pro* (for, before) and *polis* (city, community) as a description of a substance used in the defence of the hive [1]. The mechanical properties of propolis are used by bees to cover hive walls, to fill hive cracks or gaps and to embalm killed invaders that cannot be transported out of the hive [1]. But propolis is also responsible for antimicrobial activities that may reduce the incidence of pathologies within the hive [4,5]. Because of its biological activities, propolis has been used as a popular natural remedy since ancient times, attracting the attention of scientists for the individuation of its pharmacologically active compounds [1–5]. A number of studies attribute the pharmacological activities of propolis

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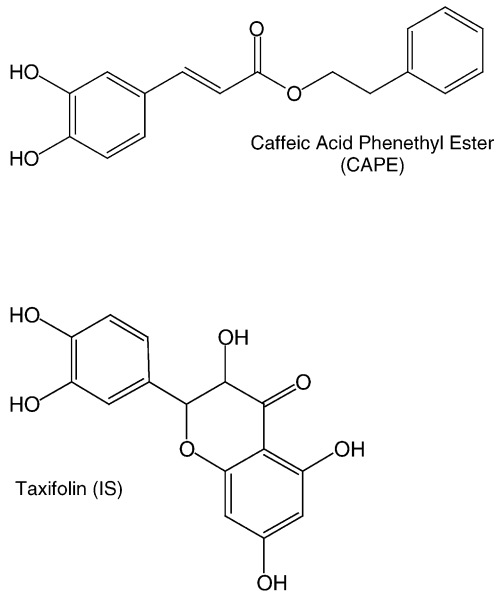

Fig. 1. Chemical structures of CAPE (M_r, 284.3) and IS (M_r, 304.3).

to flavonoids, phenolic acids and derivatives. Among these phenolic compounds, caffeic acid phenethyl ester (CAPE; Fig. 1), first described in propolis in 1987 [6], resulted one of the most interesting bioactive compounds, showing potential therapeutic relevance for the prevention of many diseases [3,7]. It has been reported that CAPE plays an important role in the antioxidant properties of propolis extracts [8] and that is able to block the production of reactive oxygen species (ROS) and to inhibit the xanthine/xanthine oxidase system [8,9]. Antinflammatory activity of CAPE has been attributed to inhibition of lipoxygenase [9] and cycloxygenase (1 and 2) activities, as well as cycloxygenase 2 gene expression [10]. Several studies reported an antitumoral activity of CAPE, by an apoptosis-induced mechanism, on different cell lines [11–13] and in $Apc^{Min/+}$ mice, which spontaneously develop multiple intestinal adenomas [14]. Although the molecular bases for the multiple activities of CAPE are controversial and not cleared, it has been reported that CAPE is a potent and highly specific inhibitor of the nuclear factor NF-κB [15]. Furthermore, CAPE has shown an interesting anti-microbial activity inhibiting bacterial motility [16] and human immunodeficiency virus integrase [17], and preventing herpes simplex virus infection [18].

Analytical methods used for the determination of bioactive polyphenols have been recently reviewed [19]. Different analytical methods have been used in several profiling studies investigating the chemical composition of the polar fraction of propolis, usually related to the biological activities. These methods mainly involved chromatographic techniques, such as capillary electrophoresis [20,21] and RP-HPLC with DAD [22–26] and mass spectrometry detection, either by ESI with an ion trap [26] or APCI with a magnetic sector [27]. APCI–ion trap mass spectrometry was also directly used without HPLC separation for propolis characterisation [25]. Anyway, the most used technique was GC with mass spectrometric detection after derivatisation [6,22–24,28–30].

CAPE has been identified in propolis by GC–MS [6,28-30], and quantified only by GC–FID [31] and LC–MS in propolis [32] and by HPLC–UV in a propolis-containing gel [33].

Recently, an increasing number of in vivo studies involving a systemic administration of CAPE in rats and mice have been described [34–37], but, to our knowledge, specific methods for CAPE quantification in biological matrices have not yet been reported.

In this study we report on the development and validation of a LC–ESI–MS/MS method for the determination of CAPE in rat plasma and urine after oral administration.

2. Experimental

2.1. Chemicals and materials

Caffeic acid phenethyl ester (CAPE) (>97%), taxifolin (>85%), ultrapure β-glucuronidase Type X-A (E.C. 3.2.1.31; G7896) and sodium azide were purchased from Sigma (St. Louis, MO, USA). Sodium fluoride was obtained from Fluka (Buchs, Switzerland). Sodium heparin (Liquemin® 5000 U.I./ml) was purchased from Roche (Milan, Italy) and pentobarbital sodium from Siegfried CMS (Zofingen, Switzerland). Transcutol® HP (diethylene glycol monoethyl ether) was gently provided by Gattefossé Italia (Milan, Italy). Deionised water was obtained from Laboratori Diaco Biomedicali (Trieste, Italy) and acetonitrile, ethyl acetate and methanol (HPLC grade), and all other chemicals (analytical grade) were purchased from Carlo Erba Reagenti (Milan, Italy). CAPE-free plasma and urine were obtained in our laboratories from untreated male CD cobs strain rats (Charles River Laboratories Italia, Calco, Lecco, Italy).

2.2. Standard solutions

Standard stock solutions of CAPE and the internal standard (IS) taxifolin were prepared by dissolving the standards in methanol at the final concentration of 1 mg/ml.

The IS was diluted in methanol/water (50:50, v/v) obtaining an IS working solution at the concentration of 1 μg/ml. Standard solutions were stored at −20 °C until use.

10 μg/ml CAPE working solutions were prepared, just before their use, in rat blank plasma (containing 0.25% sodium fluoride and 0.1 M acetate buffer pH 5.0) and rat blank urine (containing 0.05% sodium azide and 0.1 M acetate buffer pH 5.0).

2.3. Instrumentation and operating conditions

A Perkin-Elmer 200 micro LC pump system (Norwalk, CT, USA) was used for the chromatographic separations. Analyses were performed at room temperature using a RP Luna C18(2) column (250 × 3.0 mm; 5 μm, 100 Å) provided

with a SecurityGuard™ System with RP Luna C18(2) cartridge (4.0 mm × 2.0 mm; 5 μm, 100 Å), both purchased from Phenomenex (Torrance, CA, USA). Samples were automatically injected using a Perkin-Elmer 200 autosampler (thermostated at 4 °C) equipped with a 20 μl sample loop and separations were carried out using a linear gradient of acetonitrile (A) in water (B), both with 0.5% formic acid (15% A for 2 min; then to 80% A in 5 min and to 100% A in 2 min; at 100% A for 5 min) at the flow rate of 0.5 ml/min.

A Sciex API 365 triple–quadrupole mass spectrometer (Toronto, Canada) was used as detector system. The mass spectrometer was calibrated with polypropylene glycol (PE Sciex, Foster City, CA, USA) and the resolution was set at 0.7 ± 0.1 u (as peak width at half height). Instrument control and data acquisition were performed with a Power Macintosh 7600/132 (Apple, Cupertino, CA, USA) using Masschrom 1.1.1 software (PE Sciex).

The HPLC system was connected to the mass spectrometer through a Sciex turboionspray source after flow splitting (1:1) and mass spectrometric parameters were optimised by infusion at the flow rate of 5 μl/min of CAPE and IS standard solutions (10 μg/ml in water/methanol, 50:50 v/v) in the mobile phase (50% A) using a "make-up" system and a model 11 syringe pump (Harvard Apparatus, South Natick, MA, USA).

Analyses were performed in negative ion mode by multiple reaction monitoring (MRM) and the following precursor to product ion transitions were used: *m/z* 283→135 for CAPE and *m/z* 303→285 for the IS. The nebulizer gas flow (air) and the curtain gas flow (nitrogen) were both set at 2.7 l/min. The turboprobe was heated at 400 °C with the auxiliary gas flow (nitrogen) set at 6 l/min. The turboionspray potential was set at −4000 V for both CAPE and IS, whereas the orifice was set at −31 and −21 V, and the ring at −230 and −200 V for CAPE and IS, respectively. The collisionally-activated dissociation gas pressure (nitrogen) was maintained at 0.36 Pa, whereas the collision energies were −31 eV (CAPE) and −15 eV (IS). The dwell time was set at 600 ms for both CAPE and IS.

2.4. Extraction procedure

Two hundred microlitres of sample (plasma or urine) were transferred to a 15 ml polypropylene conical tube with the addition of 50 μl of the IS working standard solution and 500 μl of 10 mM HCl. The extraction was performed by adding 3 ml ethyl acetate. After 15 min vortexing followed by 15 min centrifugation at 3000 × *g* in a benchtop centrifuge, the organic phase was transferred to a new 15 ml polypropylene conical tube, evaporated to dryness under nitrogen flow and stored at −20 °C. Samples were reconstituted just before analysis with 200 μl of water/methanol (50:50, v/v) and, after 15 min vortexing and 15 min centrifugation at 3000 × *g* to remove any particulate material, transferred to autosampler microvials to be injected and analysed. Before deproteinization, all plasma samples were handled in ice.

2.5. Method validation

Blank plasma and urine were spiked with CAPE working solutions to obtain standard curves ranging from 1000 to 5 ng/ml. Lower concentrations were also prepared for the determination of the detection limit. To evaluate the linearity of the method, six independent calibration curves for both plasma and urine were prepared and analysed.

Peak integration of extracted ion chromatograms (*m/z* 283→135 for CAPE and *m/z* 303→285 for IS) and all calculations of concentrations and regression parameters were performed using PE Sciex TurboQuan 1.0 software.

Calibration curves were calculated using weighted (*1/y*) linear regression of internal ratios (CAPE/IS peak areas) versus CAPE concentrations. After back-calculation of concentrations from the regression curve, precision and accuracy were expressed as relative standard deviation (R.S.D.%) and relative bias (Bias%), respectively, of the back-calculated concentrations.

The range of the method was assessed as the interval of concentrations in which the correlation coefficient (*r*) was ≥0.985 and precision and accuracy were ≤15%, except for the lower limit of quantitation (LLOQ), the lowest quantifiable concentration for which precision and accuracy ≤20% were accepted. The limit of detection (LOD) was considered as the lowest concentration that can be discriminated from the baseline level with signal intensity at least three times greater than the background level.

The intra-batch and inter-batch variability of the method were evaluated by analyses of quality control (QC) samples spiked at three different concentrations (7.5, 75 and 750 ng/ml). Three different batches of six replicates of each QC were prepared and analysed. The intra-batch assay was performed with the first batch, whereas the inter-batch assay was performed with the three batches together.

The recovery of the extraction procedure was estimated for CAPE at the three different concentrations of QC samples (n = 6) by comparing the peak areas of blank samples spiked before and after liquid/liquid extraction. The recovery of IS was estimated as for CAPE at the amount used in the extraction procedure (50 ng; n = 18). The repeatability of the extraction procedure was assessed as R.S.D.% of the peak areas.

Matrix effects, probably due to endogenous components interfering with the ionisation efficiency, were evaluated by spiking the extracts of six different blank samples (BS) and the reconstitution solvent (RS) at the QC concentrations for CAPE and at the amount used in the extraction procedure (50 ng) for IS, then comparing the peak areas as follows: [(area BS/area RS) × 100]−100.

2.6. Stability

The stability of CAPE was evaluated at different time intervals (1–24 h), at room temperature (20–22 °C) and at 37 °C, by spiking blank plasma and urine at the

concentrations of 7.5 and 750 ng/ml and using no additives. Stability was then evaluated in plasma (for 1 h in ice) after addition of sodium fluoride and acetate buffer pH 5.0 to a final concentration of 0.25% and 0.1 M, respectively, and in urine (from 1 to 24 h at 20–22 °C) after addition of sodium azide and acetate buffer pH 5.0 to a final concentration of 0.05% and 0.1 M, respectively. Stability after three freeze and thaw cycles and after 1 month storage at −20 °C were also assessed using additives, as described above. Stability was expressed as recovery (%) of CAPE by comparing the concentrations of stored samples with those of freshly prepared samples (control samples). Furthermore, autosampler stability was assessed by reanalysing the processed QC samples 24 and 48 h after the first injection ($n = 4$). The repeatability was assessed by R.S.D.% of the calculated concentrations.

2.7. Method application

Male CD cobs rats weighing 300 ± 50 g were housed in a temperature-controlled room (21 ± 2 °C) with 12 h of daily artificial illumination. Food and water were given ad libitum. Experimentation was conducted in compliance with European laws and policies [38]. After 1-week acclimatation, rats were treated by gavage with CAPE (30 mg/ml) dissolved in the vehicle (Transcutol® HP/water/ethanol, 45:45:10, v/v/v) at the dose of 100 mg/kg. After anaesthesia with pentobarbital sodium given by intra-peritoneal administration (40 mg/kg), blood (5 ml) was withdrawn by cardiac puncture at 15, 30, 60, 120 and 360 min after treatment, and collected in tubes containing 10 μl heparin, 500 μl 2.5% sodium fluoride and 250 μl 2 M acetate buffer pH 5.0. Plasma, obtained by centrifugation at 3000 × *g* for 15 min at 4 °C, was stored at −20 °C until analysis. For the excretion study, treated rats were put in metabolic cages and urine were collected in glasses containing 20 μl 25% sodium azide and 500 μl 2 M acetate buffer pH 5.0, for 4 days at 24 h intervals. Urine were centrifuged at 3000 × *g* for 15 min at 4 °C to remove particulate material and stored at −20 °C until analysis. For both plasma and urine studies, control samples were obtained by treating rats with the vehicle alone.

In order to investigate the possible formation of CAPE glucuronide conjugate, 200 μl aliquots of plasma and urine were both mixed with 50 μl phosphate buffer (1 M, pH 6.8) containing β-glucuronidase (800 U/ml) and incubated for 60 min at 37 °C. After the enzymatic treatment, samples were extracted as described in Section 2.4.

3. Results and discussion

3.1. Mass spectrometry and liquid chromatography

CAPE, as well as other polyphenols, can be easily analysed by mass spectrometry in ESI negative ion mode. Formic acid and ammonium formate were tested as modifiers in order to increase sensitivity. Addition of 0.5% formic acid and 20 mM ammonium formate have shown to enhance sensitivity at least eight-fold and four-fold, respectively, as compared to solvent with no additives. Typical MS/MS fragmentation patterns for CAPE and IS are shown in Fig. 2. The most abundant fragmentation ions for CAPE are due to the ester cleavage, resulting in the formation of caffeic acid ion (*m/z* 179) and of its decarboxylated form (*m/z* 135). The main fragmentation product for the IS is due to the loss of water (*m/z* 285) from the $[M–H]^-$ ion. Product ions *m/z*, 135 and *m/z* 285 were then chosen for CAPE and IS MRM detection, respectively.

Chromatographic conditions for the separation of both CAPE and IS were optimized by modifying a previously described method [32]. Representative chromatograms of plasma and urine samples are shown in Figs. 3 and 4.

APCI was also tested, but sensitivity and specificity were substantially the same obtained with ESI. However, APCI was not used since it has shown a non-linear detector response during preliminary tests in the same concentration range used with ESI.

Sample clean-up is based on a liquid/liquid extraction, optimised by testing different organic solvents (methanol, acetonitrile, ethyl acetate, diethylether), different solvent volumes, the influence of the pH and of single or double extraction on the recovery. Purification by SPE with C18 and

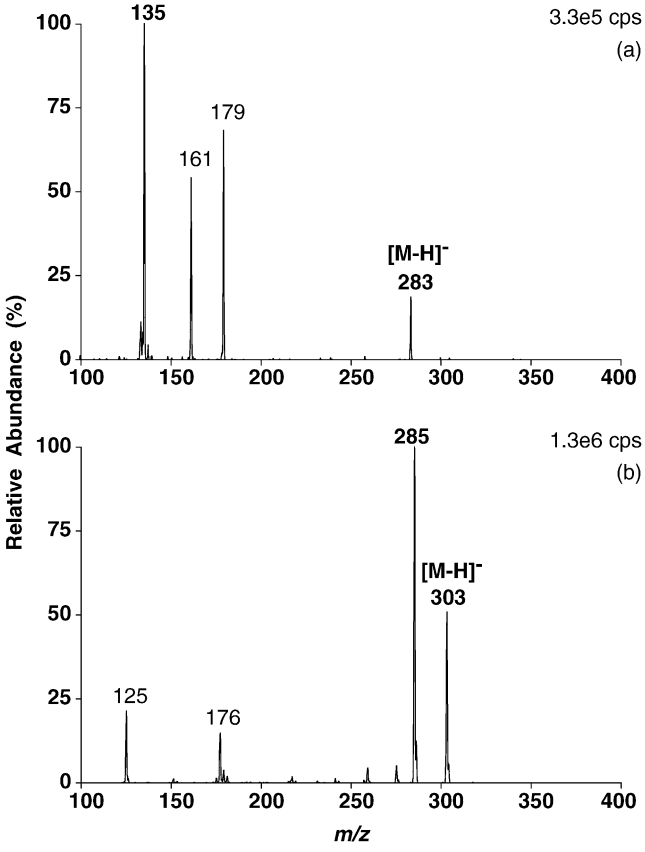

Fig. 2. Product ion mass spectra of (a) CAPE and (b) taxifolin (IS) obtained by infusion of the standards (see Section 2.3 for details).

polymeric sorbents was also tested, but an unsatisfactory recovery of CAPE from the matrices was obtained (data not shown). Anyway, the best results were obtained with the procedure described in Section 2.4. Selectivity was evaluated by comparing the extracted chromatograms of blank samples of both matrices from six different untreated rats with those of the same matrices spiked with CAPE at the LLOQ concentration and IS at the amount used in the procedure, and no peaks interfering with CAPE and IS determination were observed. Since a little carry-over effect was observed for CAPE during the analyses of the biological matrix extracts, a longer column-washing step (100% A for further 5 min) was used and an additional solvent injection was done after the analysis of high concentrated samples, in order to minimize carry-over, which thus results negligible in CAPE determination.

3.2. Linearity, precision, accuracy and recovery

As shown in Table 1, the method was linear ($r \geq 0.9968$) over the range 5–1000 ng/ml, with precision and accuracy $\leq$ 12.7% for both plasma and urine. The LOD was 1 ng/ml in both matrices. The intra- and inter-batch assays (Table 2) have shown good repeatability and accuracy ($\leq$ 14.5 and $\leq$13.4%, respectively) of the method. Good recoveries in both plasma and urine, ranging from 94 to 106% (R.S.D.% $\leq$ 17.1%) for CAPE and from 104 to 109% (R.S.D.% $\leq$ 14.1%) for the IS, were obtained with a single and relatively fast liquid/liquid extraction. Moreover, a signal enhancement due to a positive matrix effect, ranging from 8 to 19% (R.S.D.% $\leq$ 13.2%), was observed for both CAPE and IS in both plasma and urine samples.

3.3. Stability

Stability has been preliminarily tested on matrices with no additives. As shown in Table 3, unexpected dramatic disappearance of CAPE, enhanced at 37 °C, was observed in plasma and, in a lesser amount, also in urine. As described for other aryl-esters, an enzymatic hydrolysis probably occurs due to the action of plasmatic esterases. To avoid further enzymatic degradation after blood collection, sodium fluoride, a reversible esterase inhibitor, has been used during sampling [39]; moreover, samples were handled in ice until the extraction/deproteinization step. As shown in Table 4, using these conditions CAPE resulted stable at least for the time of sample preparation ($\leq$1 h). Instability of CAPE in urine (Table 3) was probably due to microbial degradation

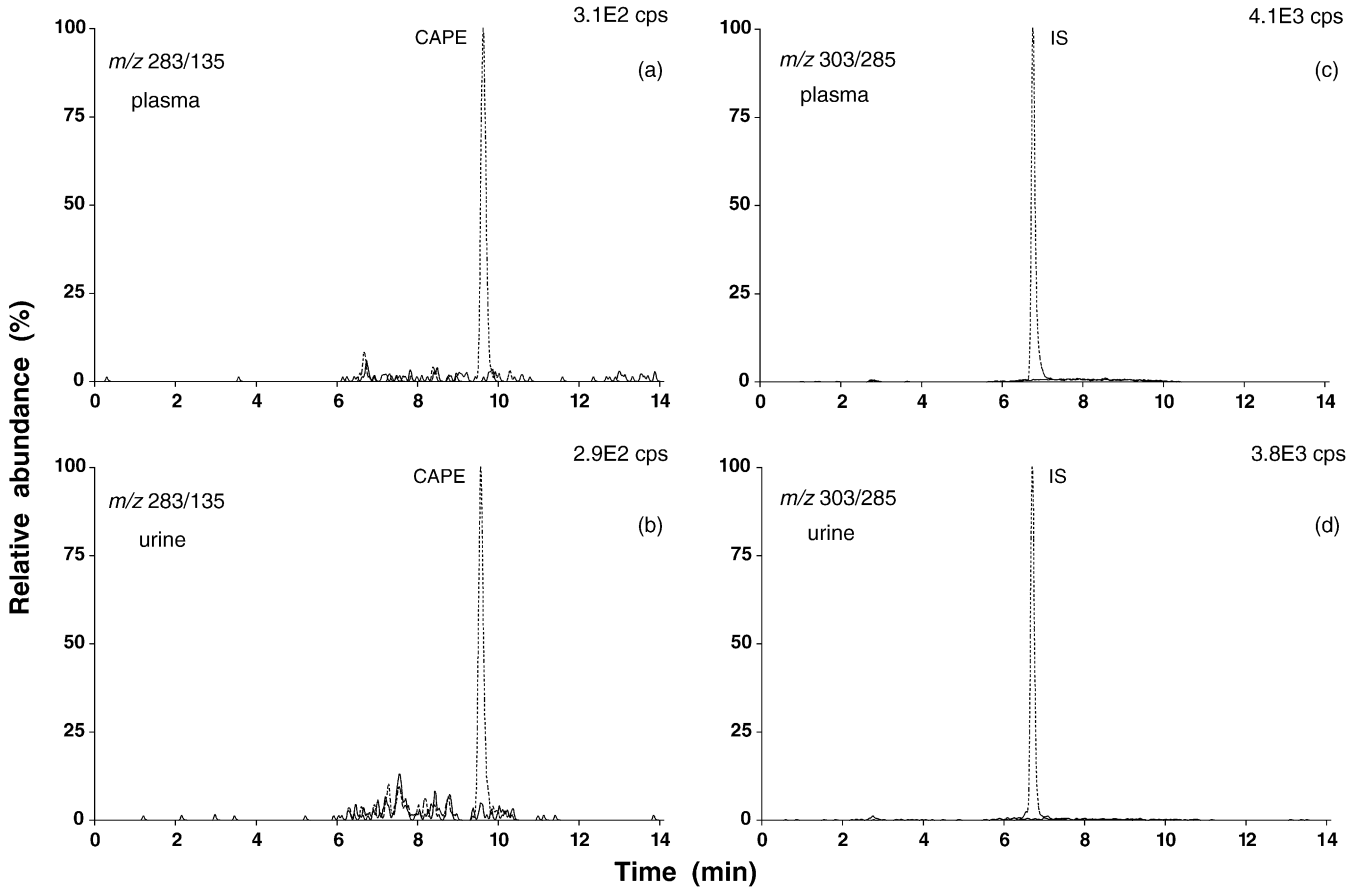

Fig. 3. Representative LC–MRM chromatograms (*m/z* 283/135 for CAPE and *m/z* 303/285 for IS) of unspiked blank matrices (solid lines) and blank matrices after spiking (dotted lines) with CAPE at LLOQ (5 ng/ml) in (a) plasma and (b) urine, and with the IS (50 ng) in (c) plasma and (d) urine.

Table 1
Linearity, precision (R.S.D.%) and accuracy (Bias%) of CAPE in spiked blank plasma and urine (n = 6)

Nominal concentration (ng/ml)	Plasma			Urine		
	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D.%	Bias%	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D.%	Bias%
5.000	5.148 ± 0.653	12.7	3.0	5.435 ± 0.430	7.9	8.7
10.00	9.641 ± 1.133	11.7	−3.6	10.75 ± 0.34	3.2	7.5
25.00	25.39 ± 1.65	6.5	−1.6	24.25 ± 2.89	11.9	−3.0
50.00	52.71 ± 3.99	7.6	5.4	46.91 ± 2.84	6.1	−6.2
100.0	102.8 ± 9.2	11.6	2.8	93.32 ± 7.17	7.7	−6.7
250.0	235.3 ± 11.6	4.9	−5.9	229.3 ± 14.6	6.3	−8.3
500.0	487.4 ± 28.0	5.7	−2.5	497.6 ± 46.9	9.4	−0.5
1000	1027 ± 20	2.0	2.7	1044 ± 40	3.8	4.4
Slope	0.0218 ± 0.0028			0.0395 ± 0.0047		
Intercept	0.0533 ± 0.0297			−0.1098 ± 0.0345		
r	0.9984 ± 0.0006			0.9968 ± 0.0016		

Table 2
Precision (R.S.D.%) and accuracy (Bias%) in intra- and inter-batch assays of CAPE in spiked blank plasma and urine samples

Nominal concentration (ng/ml)	Intra-batch assay[a]			Inter-batch assay[b]		
	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D.%	Bias%	Observed concentration (ng/ml) (mean ± S.D.)	R.S.D.%	Bias%
Plasma						
7.50	8.36 ± 0.47	5.7	11.4	7.22 ± 1.05	14.5	−3.8
75.0	77.9 ± 1.9	2.5	3.9	71.8 ± 6.9	9.7	−4.3
750	750 ± 57	7.5	−0.1	698 ± 64	9.1	−6.9
Urine						
7.50	7.80 ± 0.44	5.6	4.0	8.37 ± 0.62	7.4	11.5
75.0	79.0 ± 6.0	7.6	5.3	79.2 ± 5.8	7.3	5.6
750	851 ± 55	6.4	13.4	791 ± 75	9.5	5.5

[a] n = 6.
[b] n = 18.

Table 3
Stability of CAPE in spiked rat plasma and urine at room temperature (RT) and 37 °C, expressed as mean recovery% (R.S.D.%) (n = 4)

Time	7.5 ng/ml		750 ng/ml	
	RT	37 °C	RT	37 °C
Plasma				
0 (control)	100.0 (8.4)		100.0 (4.0)	
1 h	BLOQ	ND	29.2 (8.2)	6.2 (2.8)
6 h	ND	ND	6.2 (10.3)	1.5 (6.3)
24 h	ND	ND	1.3 (7.2)	ND
Urine				
0 (control)	100.0 (1.7)		100.0 (3.6)	
1 h	94.7 (1.9)	85.2 (5.8)	94.4 (5.5)	93.8 (3.0)
6 h	84.9 (4.9)	BLOQ	71.6 (2.1)	22.9 (4.2)
24 h	BLOQ	BLOQ	30.0 (5.0)	7.4 (8.2)

BLOQ: below (lower) limit of quantitation; ND: not detectable.

and/or basic hydrolysis due to bacterial growing, since the addition of sodium azide and acetate buffer prevented the degradation of CAPE in urine (Table 4). No significant degradation of CAPE has been observed after three freeze and thaw cycles and after 4 weeks storage at −20 °C in both matrices (Table 4). After resuspension of the extracted samples, CAPE was stable for at least 48 h in the autosampler (data not shown).

3.4. Method application

The method was applied to the determination of CAPE in rat plasma and urine after oral administration (see Section 2.7 for details). Typical chromatograms of treated rat samples are shown in Fig. 4.

As shown in Fig. 5a, CAPE was rapidly absorbed, showing the maximum plasma level at (or before) 15 min with

Table 4
Stability of CAPE in spiked rat plasma (containing sodium fluoride and acetate buffer) in ice, and in spiked rat urine (containing sodium azide and acetate buffer) at room temperature[a]

Time	Plasma		Urine	
	7.5 ng/ml	750 ng/ml	7.5 ng/ml	750 ng/ml
0 (control)	100.0 (4.9)	100.0 (7.6)	100.0 (5.7)	100.0 (8.6)
1 h	108.8 (6.4)	105.1 (4.1)	95.7 (6.6)	90.1 (4.9)
6 h			98.4 (6.4)	89.2 (4.1)
24 h			102.8 (2.4)	93.5 (3.5)
3 freeze and thaw cycles	114.5 (3.5)	112.8 (0.9)	99.1 (3.7)	104.4 (4.5)
4 weeks (−20 °C)	90.4 (9.7)	91.3 (6.4)	92.0 (2.9)	109.3 (8.0)

[a] Results are expressed as mean recovery% (R.S.D.%) (n = 4).

a mean value of 18.0 ± 6.2 ng/ml. When treated with β-glucuronidase, the same plasma samples showed increased levels of CAPE at all the monitored times (Fig. 5b) ranging from 129 ± 12 ng/ml (60 min) to 61.2 ± 7.4 ng/ml (120 min).

As shown in Fig. 6, 93% of the recovered CAPE was eliminated in urine within the first 24 h after the treatment, both as glucuronide conjugate (60%) and unmodified molecule (40%) and the percentage of the administered dose recovered

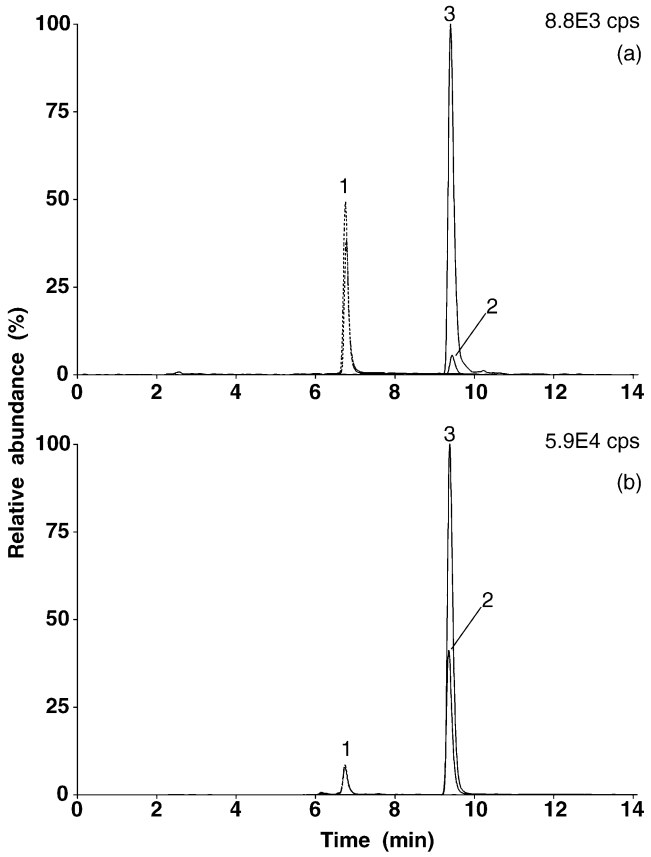

Fig. 4. Representative LC–MRM chromatograms of plasma and urine samples of rats treated with CAPE 100 mg/kg, obtained by superposition of the extracted ion chromatograms m/z 283/135 for CAPE (solid lines) and m/z 303/285 for the IS (dotted lines): (a) plasma sample (15 min after the treatment); (b) urine sample (collected within 24 h after the treatment). Peak 1, IS; Peak 2, CAPE before incubation with β-glucuronidase; Peak 3, CAPE after incubation with β-glucuronidase.

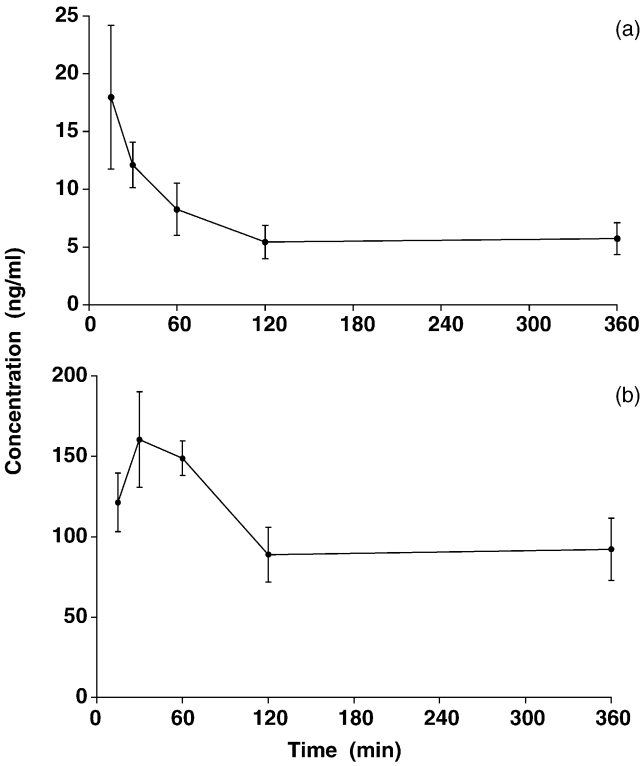

Fig. 5. Concentration–time profiles of CAPE in rat plasma: (a) before and (b) after incubation with β-glucuronidase (n = 3).

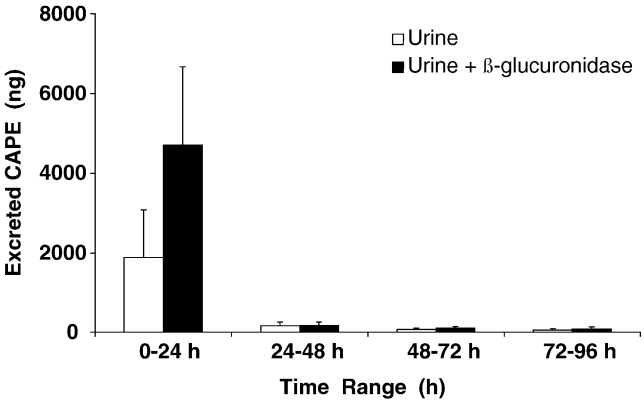

Fig. 6. Excretion of CAPE in rat urine before and after incubation with β-glucuronidase (n = 5).

up to 96 h varied from 0.007 to 0.021%. The low plasma and excretion levels of CAPE (when compared with the administered dose) can be partially explained as a result of its rapid hydrolysis in plasma (see Section 3.3), even if the early appearance of the glucuronide conjugate does not allow excluding other metabolic pathways. Anyway, further studies to better investigate the metabolic fate of CAPE are currently ongoing in our laboratories.

4. Conclusions

In this study, a LC–ESI–MS/MS method for the determination of CAPE in rat plasma and urine has been described for the first time. The method resulted sensitive, selective, accurate and repeatable. The application of the method to a bioavailability study in rats after oral administration of CAPE has shown a rapid adsorption even if low plasma levels were found. CAPE was rapidly excreted both as unmodified molecule and as glucuronide conjugate, but in a low amount if compared with the administered dose. Low concentrations found in plasma and urine may be partially attributed to the rapid hydrolysis of CAPE by plasmatic esterases, but a low absorption of CAPE in the gastrointestinal tract cannot be excluded. Anyway, the method has shown to be suitable for CAPE pharmacological and pharmacokinetic investigations and, if opportunely improved, it may also be applied to other matrices and administration routes.

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