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Journal of Chromatography B, 758 (2001) 249–264

JOURNAL OF CHROMATOGRAPHY B

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High-performance liquid chromatography method for the quantification of non-radiolabelled cinnamic compounds in analytes derived from human skin absorption and metabolism experiments

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Received 3 November 2000; received in revised form 20 March 2001; accepted 20 March 2001

Abstract

An isocratic high-performance liquid chromatography method has been developed for the quantification of the skin sensitisers *trans*-cinnamaldehyde and *trans*-cinnamic alcohol, and their cinnamic metabolites. The relative standard deviations (RSDs) between the gradients of eight sets of standard curves were 2.8, 3.1 and 1.9% for cinnamic alcohol, cinnamaldehyde and cinnamic acid, respectively. Sample analytes were derived from two series of experiments: in vitro full-thickness human skin absorption and metabolism studies and metabolism studies using human skin homogenates, with non-radiolabelled cinnamic compounds. Skin absorption and metabolism experiments were performed in the absence and presence of the alcohol dehydrogenase inhibitor, pyrazole. Samples from full-thickness skin absorption studies were analysed without extraction; cinnamic compounds from within skin were extracted into methanolic solutions using newly developed methods. The intra-assay RSDs ranged from 0.17 to 2.52% for cinnamic alcohol, 0.24 to 9.14% for cinnamaldehyde and 0.26 to 6.43% for cinnamic acid. The inter-assay RSDs for cinnamic alcohol, cinnamaldehyde and cinnamic acid, respectively, as determined from $n=20$ HPLC runs, were 2.10, 4.16 and 2.26%.

Keywords: Cinnamic aldehyde; Cinnamic alcohol; Cinnamic acid

1. Introduction

trans-Cinnamaldehyde and *trans*-cinnamic alcohol are the two major components of the commonly used and naturally occurring fragrance and flavouring agent cinnamon. Skin absorption of cinnamaldehyde or cinnamic alcohol, as a result of exposure either at work or in the home, can result in allergic contact dermatitis (ACD) and skin irritation in humans [1–12]. Consequently, accurate measurements of the extent of cinnamaldehyde and cinnamic alcohol skin absorption and metabolism are of interest to both dermatologists and risk assessors working in the food and fragrance industries.

Cinnamaldehyde is a more potent skin sensitiser than cinnamic alcohol, as determined by in vivo and in vitro tests [5,13–15]. In some individuals, even low levels (5000 ppm in consumer products and 0.5% in petrolatum for patch test studies) of cinnamaldehyde can elicit ACD [1,3], presumably by

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00184-0

acting as a hapten by binding to host protein/peptide to form an immunogen [16,17]. The lowest dose of cinnamic alcohol that has been seen to elicit ACD in humans is 4% in petrolatum [5].

It is known that protein-reactive cinnamaldehyde and cinnamic alcohol (not protein-reactive) can be metabolised in human skin (Fig. 1) [18–20]. It is hypothesised that the cutaneous oxidoreductase enzyme, alcohol dehydrogenase (ADH; E.C. 1.1.1.1) [21], performs the interconversion of cinnamic alcohol and cinnamaldehyde, that latter of which acts as the hapten in cinnamic alcohol-derived ACD [14,18,19]. Cinnamaldehyde may also be oxidised by cutaneous aldehyde dehydrogenase (E.C. 1.2.1.3) [21] to cinnamic acid (Fig. 1), which is non-sensitising [13]. However, at high doses (>5%, w/v, in petrolatum) cinnamaldehyde and cinnamic acid can act as skin irritants [9]. Hence, very low residual levels of cinnamaldehyde within the skin (following exposure, absorption and metabolism of cinnamic compounds) can lead to skin sensitisation and high residual levels may lead to skin irritation. Therefore,

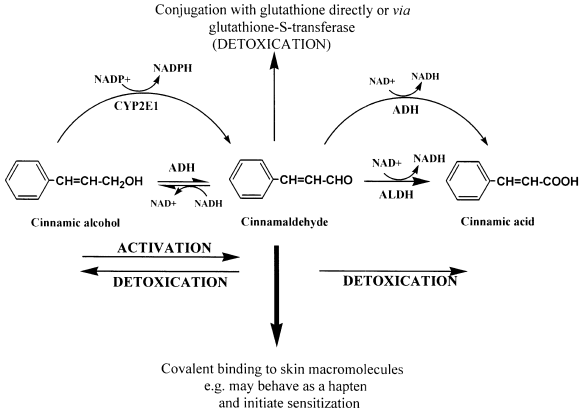

Fig. 1. Proposed metabolism of cinnamaldehyde and cinnamic alcohol. Cinnamaldehyde (formula weight, FW=132.16 g/mol) is an α,β-unsaturated aromatic aldehyde. Cinnamic alcohol (FW=134.16 g/mol) is an aromatic alcohol and is the reduced form of cinnamaldehyde. The interconversion of cinnamaldehyde and cinnamic alcohol can be catalysed by alcohol dehydrogenase (ADH). Cinnamaldehyde can be irreversibly oxidised to cinnamic acid (FW=148.16 g/mol) by either aldehyde dehydrogenase (ALDH), or ADH acting as a dismutase with NAD^+ as cofactor. Cinnamaldehyde may also bind to skin proteins or glutathione. This figure has been accepted for publication [18].

it is important to quantify accurately the levels of cinnamic compounds that penetrate through and remain within human skin following exposure.

Previously, cinnamic compound penetration through human skin has been quantified crudely by gas chromatography (GC) [22] and high-performance liquid chromatography (HPLC) [20] methods, in analytes derived from static diffusion cell skin absorption model systems. Jimbo [22] used a complicated extraction procedure, involving large volumes (50 ml) of ether, sodium sulfate to dehydrate the ether extracts, paper filtration and condensation, to isolate low levels (nmol–μmol) of moderately volatile cinnamic compounds that had penetrated the skin into a phosphate buffered (pH 7.4) receptor fluid. Weibel and Hansen [20] used a HPLC method to analyse μmol of cinnamic compounds diluted in a relatively large volume (10 ml) of phosphate buffer (pH 7.4). However, statistical quantification was not performed against accurate standard curve data. Also, these authors were not able to observe any low level metabolite production (<μmolar range). Typically, activity of cutaneous enzymes is low in comparison to the other organs [23] and consequently the levels of metabolites generated in these previous studies would be in the order of nmol and presumably below the limit of detection using their methods.

Here, we present a new HPLC method developed to quantify nmol of non-radiolabelled cinnamic compounds in aqueous and methanolic analytes, derived from two series of experiments, either from 2 h and 24 h fresh full-thickness human skin absorption and metabolism studies or following extraction from human skin homogenate studies. In the first series, using an in vitro flow-through diffusion cell skin absorption model to collect all analytes, we have detected and quantified the levels of parent cinnamic compound and cinnamic metabolites that penetrated through the skin into a physiological receptor fluid, remained within the skin, evaporated from the skin surface or remained unabsorbed on the skin surface. A new method for the simple extraction of cinnamic compounds from within the cinnamaldehyde- and cinnamic alcohol-treated full-thickness skin samples, into a methanolic solution for direct analysis and quantification using the specifically developed HPLC method, is also described. Pre-

treatment of the skin samples with the alcohol dehydrogenase inhibitor, pyrazole, was also investigated. In the second series of experiments, we have also shown the applicability of this HPLC method for the analysis of extracted cinnamic metabolites generated by cinnamaldehyde and cinnamic alcohol treatment of human skin homogenate conducted in order to asses the metabolic capacity of skin in the absence of the absorptive barrier.

2. Experimental

2.1. Chemicals

Cinnamaldehyde (>99% purity), cinnamic alcohol (>98.9%), cinnamic acid (>98%), pyrazole (>98%), 4-methylpyrazole (>99%), benzyl alcohol (>99.9%), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), Hanks' balanced salts solution (HBSS), trifluoroacetic acid (TFA) and sodium hydrogencarbonate were purchased from Sigma–Aldrich (Gillingham, UK). Gentamycin (10 mg/ml) was purchased from Gibco BRL (Paisley, UK). HPLC-grade methanol (BDH–Merck, Poole, UK) was used throughout. Skin contact with cinnamaldehyde and cinnamic alcohol should be avoided as both molecules are skin sensitisers.

2.2. Preparation of human skin samples

Fresh healthy human skin samples, taken from reduction mammoplasty (breast) or apronectomy (abdomen) operations, were obtained from six female individuals (ages 26–64 years) who underwent surgery at St. Mary's Hospital, London or were donors to the Stephen Kirby Skin Bank, Roehampton. All skin samples were obtained with ethical approval from the Local Research Ethics Committees of St. Mary's Hospital and Stephen Kirby Skin Bank, respectively. Within 1 h following excision, the tissue was washed in ice-cold 0.9% saline solution and cleaned manually of all fat and connective tissue. Circles of skin (1.7 cm diameter) were cut using a circular sharpened steel cutter on a plastic dissection board.

2.3. Skin absorption and metabolism studies

An in vitro skin absorption model (SAM) system was used [24], which has been described for use in skin absorption studies [25,26] and skin metabolism studies [27,28], previously. A surface area of 0.32 cm^2 remained exposed for sample application. An aqueous physiological receptor fluid [0.025 *M* HEPES, Hank's balanced salts solution (9.8 g/l), 4 m*M* sodium hydrogencarbonate, 50 mg/l gentamycin, pH 7.4; degassed and filtered using a 65-μm PTFE filter (Whatman, UK) prior to use] was used. The skin samples were allowed to equilibrate unoccluded for 1 h in the skin absorption model system with the receptor fluid flowing at 2 ml/h.

Sample treatment conditions are detailed in Table 1. Doses were measured using a 20-μl glass syringe (Scientific Glass Engineering, Australia) for maximum accuracy. All samples were occluded for the duration of the experiment. During 24 h experiments, each fraction was collected over 2 h; for 2 h experiments each fraction was collected over 15 min. At the end of the experiment, receptor fluid samples were stored at −20°C. Occluded diffusion cells were then dismantled as follows: PTFE caps were removed from the occlusion cells and placed carefully in 20 ml plastic vials containing 2 ml methanol; occlusion cells were removed and placed in 50-ml Sterilin pots containing 40 ml methanol. Each skin sample was then swabbed twice with methanol-soaked lint held by tweezers and the two swabs were placed together in a 20 ml plastic vial containing 2 ml methanol. Skin samples were removed with tweezers and placed in an empty 20-ml plastic vial. Vials containing the PTFE caps, swabs and skin were all stored immediately at −20°C. Finally, the diffusion cells were placed in the Sterilin pots containing the occlusion cells in methanol and both were soaked overnight. The cells were then removed and the methanol soaking solution stored at −20°C.

2.4. Recovery of metabolites from cinnamic compound-treated full-thickness skin

Frozen skin circles that contained absorbed cinnamic compounds were thawed in their vials by incubation for 15 min at 37°C in a water bath. Skin circles were chopped manually into small (~1 mm)

Table 1
Application of test compounds to (a) full-thickness human skin samples or (b) human skin homogenates

Sample	Test compound (10 μmol neat)	30 min pre-treatment	Duration of exposure (h)
(a) Full thickness skin			
Control	None	None	24
Vehicle control	None	20 ml water	24
2-CALD	Cinnamaldehyde	None	2
24-CALD	Cinnamaldehyde	None	24
24-CALD-80PYR	Cinnamaldehyde	80 μmol pyrazole	24
24-CALD-320PYR	Cinnamaldehyde	320 μmol pyrazole	24
2-CALC	Cinnamic alcohol	None	2
24-CALC	Cinnamic alcohol	None	24
24-CALC-80PYR	Cinnamic alcohol	80 μmol pyrazole	24
24-CALC-320PYR	Cinnamic alcohol	320 μmol pyrazole	24
(b) Skin homogenate	Test compound added to skin sample		Duration of exposure (min)
Control	None		90
Vehicle control	20 μl acetone		90
Boiled control 1	Skin (heated at 100°C for 15 min) +5 μmol cinnamaldehyde		90
Boiled control 2	Skin (heated at 100°C for 15 min)		90
	+5 μmol cinnamic alcohol		30
5-CAld-30	5 μmol cinnamaldehyde		90
5-CAld-90	5 μmol cinnamaldehyde		
5-CAld-30	5 μmol cinnamic alcohol		30
5-CAld-90	5 μmol cinnamic alcohol		90

pieces using sharp dissection scissors. Chopped skin was returned to its original vial and 4 ml of buffer A (70% methanol–50 m*M* sodium citrate, pH 5) was added. The skin samples were then homogenised on ice using an Ultra Turrax T25 (Janke & Kunkel, IKA Labortechnik) for 5×30 s bursts. This freeze–thaw step was seen to aid the homogenisation of the skin samples. The probe was washed with 2 ml of buffer A between each sample and the probe washes were kept separately in individual vials to be combined with the skin samples later. The 4 ml of each skin homogenate was frozen in liquid nitrogen, thawed quickly in a water bath at 37°C and homogenised a second time on ice for 5×30 s bursts. The probe was washed with 2 ml of buffer A between each sample as before. The homogenates were boiled for 2 min in a water bath (in the sealed vial to prevent evaporation) and cooled quickly on ice. To lyse the cells completely, each suspension was sonicated at amplitude 14 for 4×15 s bursts using a sonicating probe (Soniprep 150). Each suspension was carefully transferred from its vial to a centrifuge tube, using the 2-ml probe wash solutions to wash out the vials to ensure complete transfer, and the suspensions were centrifuged at 100 000 *g* using a Sorvall Ultracentrifuge (DuPont, Wilmington, DE, USA) for 30 min. The supernatants were removed carefully into separate 10-ml graduated test tubes using a pasteur pipette and the total recovered volume was measured to the nearest 0.1 ml. Supernatants were stored at −20°C prior to HPLC analysis.

The extraction efficiency of the method, with respect to the recovery of the individual cinnamaldehyde, cinnamic alcohol and cinnamic acid compounds, was ascertained as follows. Circles of fresh human skin ($n=3$ for each cinnamic compound plus one control sample where no cinnamic compound was applied) were chopped and homogenised in 4 ml buffer A as above. A 10-μl aliquot of a 1 *M* solution (10 μmol) of either cinnamaldehyde, cinnamic alcohol or cinnamic acid was added to each of three separate skin homogenates. Each suspension was mixed and immediately snap frozen in liquid nitrogen. The remainder of the procedure was performed

as described above for the skin samples from the absorption/metabolism experiments and the resulting supernatants were stored at −20°C until HPLC analysis was performed.

2.5. Preparation of skin homogenates for treatment with cinnamic compounds

Skin homogenates of freshly obtained untreated skin were prepared as described previously using 0.1 *M* potassium phosphate glycerol buffer, pH 7.4 [21]. The total protein content of all skin homogenates was determined using the standard method of Lowry et al. and all samples were kept on ice until required [29]. Cinnamaldehyde and cinnamic alcohol were dissolved in acetone, each to stock concentrations of 100 m*M*. Reaction mixtures (1 ml total volume using 0.1 *M* potassium phosphate glycerol buffer, pH 7.4) containing 500 μl of the original skin homogenate and 1 m*M* NAD (cofactor), were incubated as detailed in Table 1 at 37°C, for either 30 min or 90 min. The reaction was terminated by immersion in liquid nitrogen and samples were stored at −20°C for subsequent analysis.

2.6. Recovery of cinnamic compounds from treated skin homogenates

The treated skin homogenates were removed from storage at −20°C, defrosted in a water bath at 37°C (<1 min) and placed directly on ice. Samples were transferred to clean 7-ml glass vials and 2 ml of 100% methanol (HPLC grade) and two drops of 0.75 *M* HCl were added (to give a final pH of 5). The suspension was mixed vigorously, centrifuged at ~3600 rpm (Ceutaur 2 bench top centrifuge; MSE, UK) and filtered through a 0.45-μm PTFE filter (Phenomenex, UK) prior to HPLC analysis.

The extraction efficiency of the method, with respect to the recovery of the individual compounds, cinnamic aldehyde, cinnamic alcohol and cinnamic acid, was determined by treating homogenates in the same way as above, with the exception that the 2 ml of 100% methanol was added immediately following addition of the cinnamic compound, to prevent metabolism occurring.

2.7. HPLC analysis of parent cinnamic compounds and cinnamic metabolites

2.7.1. Preparation of samples

Methanolic solutions were removed from storage at −20°C and allowed to equilibrate to room temperature on the bench. For each receptor fluid, cell wash or skin homogenate supernatant sample generated from the full-thickness skin absorption studies, a 2-ml sample was accurately transferred using a P1000 Gilson pipette into a clean glass 10 ml vial. The swab samples were diluted 20-fold with HPLC-grade methanol prior to taking a 2 ml aliquot. A known concentration (1.54 μmol) of benzyl alcohol (BAlc) (20 μl from a stock solution of 7.7 mmol in methanol) was added to each 2 ml sample to act as an internal standard for the HPLC analysis. A 1-ml aliquot of each sample was then filtered through a 0.45-μm PTFE filter (Phenomenex) using a 1-ml syringe into a 2-ml glass sample vial (Chromocol), which was then sealed with a rubber septum and cap (Chromocol). For the treated skin homogenate samples, 100–400 μl of the filtered suspensions were made up to 1 ml with 100% methanol (HPLC grade) and 0.77 μmol of BAlc (10 μl from a stock solution of 7.7 mmol in methanol). The amount of BAlc (internal standard) was 15.39 nmol/20 μl per injection.

2.7.2. HPLC analysis

A Shimadzu HPLC system (Dyson Instruments, UK) equipped with two LC-6A pumps, an SCL-6B controller and an SIL-6B automatic sample injector was used for all HPLC analyses. An isocratic method was employed using a 10 cm silica-C_{18} (5 μm particle size) reversed-phase column (LiChrospher; Merck) with a mobile phase of 35% methanol in water, adjusted to pH 3 with concentrated TFA, filtered through a 0.65-μm Durapore membrane filter (Millipore) and degassed under vacuum in a Buchner flask sealed with a rubber stopper for 10–15 min. The total flow-rate was maintained at 1.0 ml/min (0.5 ml/min through each of the two pumps) over a run time of 25 min per sample. Every 20 μl of analyte injected (during both standard and sample analysis) was always superceded by a 20 μl mobile phase blank injection to verify that the column was clean between each sample analysis. Elutants were

detected using a Shimadzu SPD-6A UV detector set to 254 nm and absorbances were recorded and peak areas integrated (for all analytes except skin homogenate extracts) using a Shimadzu C-R6A Chromatopac set at attenuation 2. Data for skin homogenate extract samples were recorded and peak areas integrated using the Millennium[32] Chromatography Manager software package (Waters, Watford, UK).

2.7.3. Calibration curves

Pure standards of cinnamaldehyde, cinnamic alcohol and cinnamic acid, were diluted in 100% HPLC-grade methanol to six known concentrations ranging from 0.0 to 3.1 nmol of each chemical/20 μl sample (Table 2). Benzyl alcohol (BAlc; M_r=108.13 g/mol) was used as an internal standard. Firstly, 20 μl cinnamaldehyde, 20 μl cinnamic alcohol and 20 μl cinnamic acid were diluted in 100 ml of HPLC-grade methanol in a volumetric flask (solution A). In a separate 100-ml volumetric flask, 100 μl benzyl alcohol was diluted in 100 ml of HPLC-grade methanol (solution B). Secondly, different volumes of solution A as indicated in Table 2 and 8 ml of solution B (to generate a final concentration of 15.39 nmol/20 μl analyte) were added to 100 ml of HPLC-grade methanol in 10 new volumetric flasks to generate the required standards. The compounds were run as a mixture in order to relate to samples from the skin absorption experiment, which contained all three cinnamic compounds. The ratio of the area of the cinnamic compound peak (PkareaCC) to the area of the benzyl alcohol peak (PkareaBAlc) was calculated from the peak integration values reported on the automatic integrator output (either Shimadzu C-R6A Chromatopac or Waters Millenium[32] integration). Graphs of (PkareaCC/PkareaBAlc) vs. concentrations of the known standards were plotted separately using Microsoft Excel 97 for cinnamaldehyde, cinnamic alcohol and cinnamic acid. Linear regression analyses (performed in Excel) of these standard plots yielded values for the gradient (*m*) and *y*-axis intercept (*c*) (see Eq. (1) below) and these values were used to generate three calibration curve equations, each one specific for each cinnamic compound. Initially, three 10-point calibration curves were run on 3 separate days using three different batches of solvent and integrated on the Shimadzu C-R6A Chromatopac. This data was used to generate a mean standard curve. When data collection was automated using the Millennium software[32], standard curve data was collected at the beginning of sample runs. Seven sets of calibration curves (for each cinnamic compound mixture) were generated at the beginning of sample runs on seven different days over a period of 3 months (named standard sets A–G). Standard set H was a calibration curve run using the same equipment and conditions, a year prior to standards A–G.

Table 2
Final standard (Std) concentrations used to generate calibration curves for cinnamic alcohol (CAlc; M_r=134.16 g/mol), cinnamaldehyde (CAld; M_r=132.16 g/mol) and cinnamic acid (CAcid; M_r=148.16 g/mol)

Std	Solution A (ml)	Dilution factor	Compound in 20 μl of analyte (nmol)		
			CAlc	CAld	CAcid
1	1.0	100	0.31	0.31	0.27
2	2.0	50	0.63	0.63	0.54
3	2.5	40	0.78	0.79	0.67
4	2.8	35.71	0.86	0.88	0.76
5	3.2	31.25	0.99	1.01	0.86
6	3.6	27.77	1.12	1.13	0.97
7	4.0	25.0	1.24	1.26	1.08
8	6.0	16.66	1.86	1.89	1.62
9	8.0	12.25	2.48	2.52	2.16
10	10.0	10.0	3.10	3.15	2.70

None of the compounds were observed to react with each other and all appeared stable in methanolic buffers or receptor fluid at room temperature over a period of up to a minimum of 5 days and indefinitely at −20°C. At room temperature, over a period of weeks to months, benzyl alcohol autooxidises to benzaldehyde. Therefore, all samples were run within 24 h after preparation or stored until required at −20°C. An injection volume of 20 μl of each sample was analysed.

2.7.4. Intra-assay and inter-assay variations

To verify metabolite retention times and to monitor intra- and inter-assay variability throughout the sample analyses, 20 μl of freshly prepared standard (number 3 in Table 1) was injected at the beginning, middle and end of each HPLC run. One complete HPLC run comprised 36 half hourly injections (18 samples and 18 intermittent mobile

phase blanks), which were run over a total of 18 h. To assess intra-assay variability, the mean of the PkareaCC/PkareaBAlc ratios, from the three standard injections within each run, was calculated for 20 independent HPLC runs. The relative standard deviations (RSDs) between the peak-area ratios (for $n=3$) were determined (one for each of the 20 assays) as standard error of the mean divided by the mean. To assess inter-assay variation, the mean of the ''mean PkareaCC/PkareaBAlc ratios'' from the 20 HPLC runs was determined. The RSD was calculated as standard error of the mean divided by the mean.

Quantification of parent compounds and metabolites. The cinnamic compound (CC):BAlc peak area ratios, as calculated from the sample runs in an equivalent way to that described above for standard solutions, were used in the relevant standard equations for each compound (based on Eq. (1) below), to calculate the number of nmol (x) of each cinnamic compound in each 20 μl sample analysed and finally in nmol/ml:

$$x = [(\text{PkareaCC}/\text{PkareaBAlc}) - c]/m \quad (1)$$

These values were then converted either to nmol/cm^2 skin/h to calculate penetration rates of cinnamic compounds into receptor fluid or total mean % of the initial cinnamaldehyde or cinnamic alcohol dose (78 μmol) for evaporated (recovered from the PTFE caps), non-absorbed (remaining on the skin surface), penetrated (present in receptor fluid) or compounds recovered from skin homogenates.

3. Results

3.1. Chromatography

Typical chromatograms obtained from HPLC analysis of cinnamic compounds from these studies are shown in Figs. 2–4. Fig. 2a shows a mixture of the three parent cinnamic compounds and the internal standard (benzyl alcohol) in methanol (standard 3). Examples of chromatograms from HPLC analysis of aqueous receptor fluid samples during 24 h human skin absorption and metabolism experiments with cinnamaldehyde and cinnamic alcohol are shown in Fig. 2b–d. Fig. 2b shows a receptor fluid blank run containing no cinnamic compounds and Fig. 2c and d show the traces obtained after treatment of the skin with 78 μmol neat cinnamaldehyde and cinnamic alcohol, respectively. Examples of chromatograms obtained from HPLC analysis of supernatant following extraction of cinnamic compounds into aqueous sodium citrate–methanol buffer from skin homogenates derived from full thickness skin topically applied with cinnamaldehyde or cinnamic alcohol are shown in Fig. 3a–c. Fig. 3a shows a sodium citrate–methanol buffer blank run containing no cinnamic compounds and Fig. 3b and c show the traces obtained after extraction of cinnamic compounds from skin homogenates (generated from full thickness skin samples treated with 78 μmol neat cinnamaldehyde and cinnamic alcohol, respectively). Examples of chromatograms obtained from HPLC analysis of filtrate following extraction of cinnamic compounds into aqueous potassium phosphate–methanol buffer from skin homogenates treated with cinnamaldehyde and cinnamic alcohol, are shown in Fig. 4a–c. Fig. 4a shows a potassium phosphate–methanol buffer blank run containing no cinnamic compounds and Fig. 4b and c show traces obtained after skin homogenates were treated with 5 μmol of cinnamaldehyde and cinnamic alcohol, respectively. At the wavelength (254 nm) chosen to detect nmol of all three cinnamic compounds, noise levels were negligible, column efficiency and retention times were stable. A signal-to-noise ratio of 2 was used. In agreement with known standards, retention times were 3.75–3.86 min (BAlc), 10.51–10.79 min (cinnamic alcohol), 13.70–14.17 min (cinnamaldehyde) and 15.09–15.62 min (cinnamic acid). As determined from appropriate serial dilutions of standard 1, the limits of detection were approximately 0.07, 0.08 and 0.07 nmol/20 μl analyte for cinnamic alcohol, cinnamaldehyde and cinnamic acid, respectively.

3.2. Quantification of cinnamic compounds

3.2.1. Calibration curves

Table 3 shows 10-point calibration curves [PkareaCC/PkareaBAlc vs. concentration of standard cinnamic compound (nmol/20 μl analyte)] using mean (±SD) data for three sets of standards for (a) cinnamic alcohol, (b) cinnamaldehyde and (c)

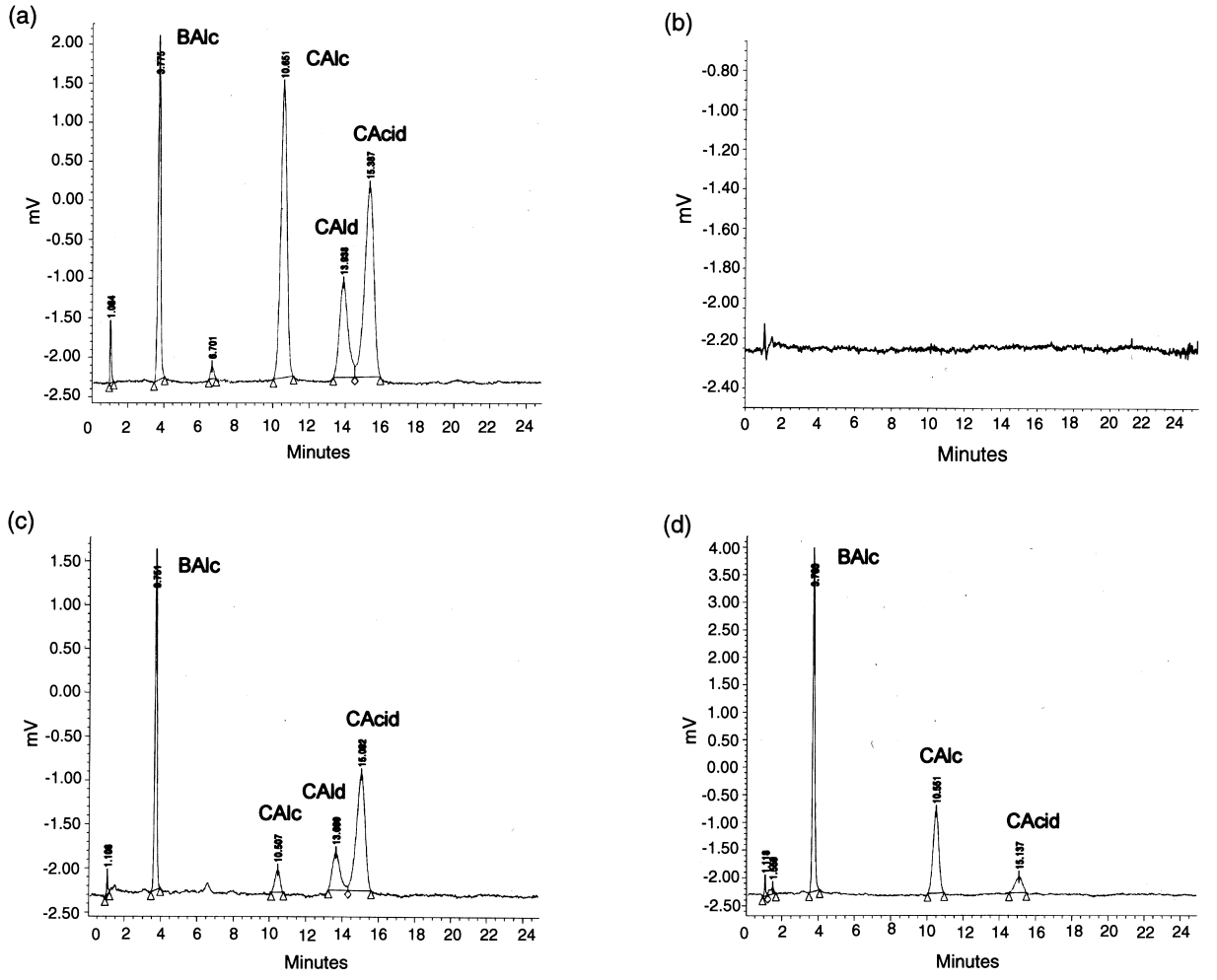

Fig. 2. Chromatographic analysis of cinnamic compounds from receptor fluid following application of cinnamaldehyde or cinnamic alcohol to human skin. Typical HPLC traces from analysis (UV detection of cinnamic compounds at 254 nm) of (a) a mixture of CAld (cinnamaldehyde), CAlc (cinnamic alcohol), cinnamic acid (CAcid) and benzyl alcohol (BAlc) standards in methanol (standard 3); (b) aqueous receptor fluid only; (c) aqueous receptor fluid fractions taken 22–24 h following topical application of 78 μmol Cald to full-thickness human skin. Retention times were 3.75–3.86 min (BAlc), 10.51–10.79 min (CAlc), 13.70–14.17 min (CAld) and 15.09–15.62 min (CAcid). Column efficiency remained stable throughout analyses, as evinced by the BAlc internal standard (15.4 nmol/20 μl sample).

cinnamic acid. The gradients, intercepts and correlation coefficients (r^2) are given in Table 3 for these three calibration curves generated from triplicate data measurements. The gradients, intercepts and correlation coefficients (r^2) are also given in Table 3, for comparison, for eight individual six-point calibration curves (including standards 1, 3, 5, 7, 9 and 10 from Table 2 and named standard sets A–G and H*) run at the beginning of sample runs over a period of a year. The RSDs between the gradients of these eight curves were 2.8, 3.1 and 1.9% for cinnamic alcohol, cinnamaldehyde and cinnamic acid, respectively. Correlation coefficients were always >0.99 for all standard curves.

3.2.2. Intra-assay variation

The RSDs, derived from $n=3$ injections performed within each of 20 HPLC runs, ranged from

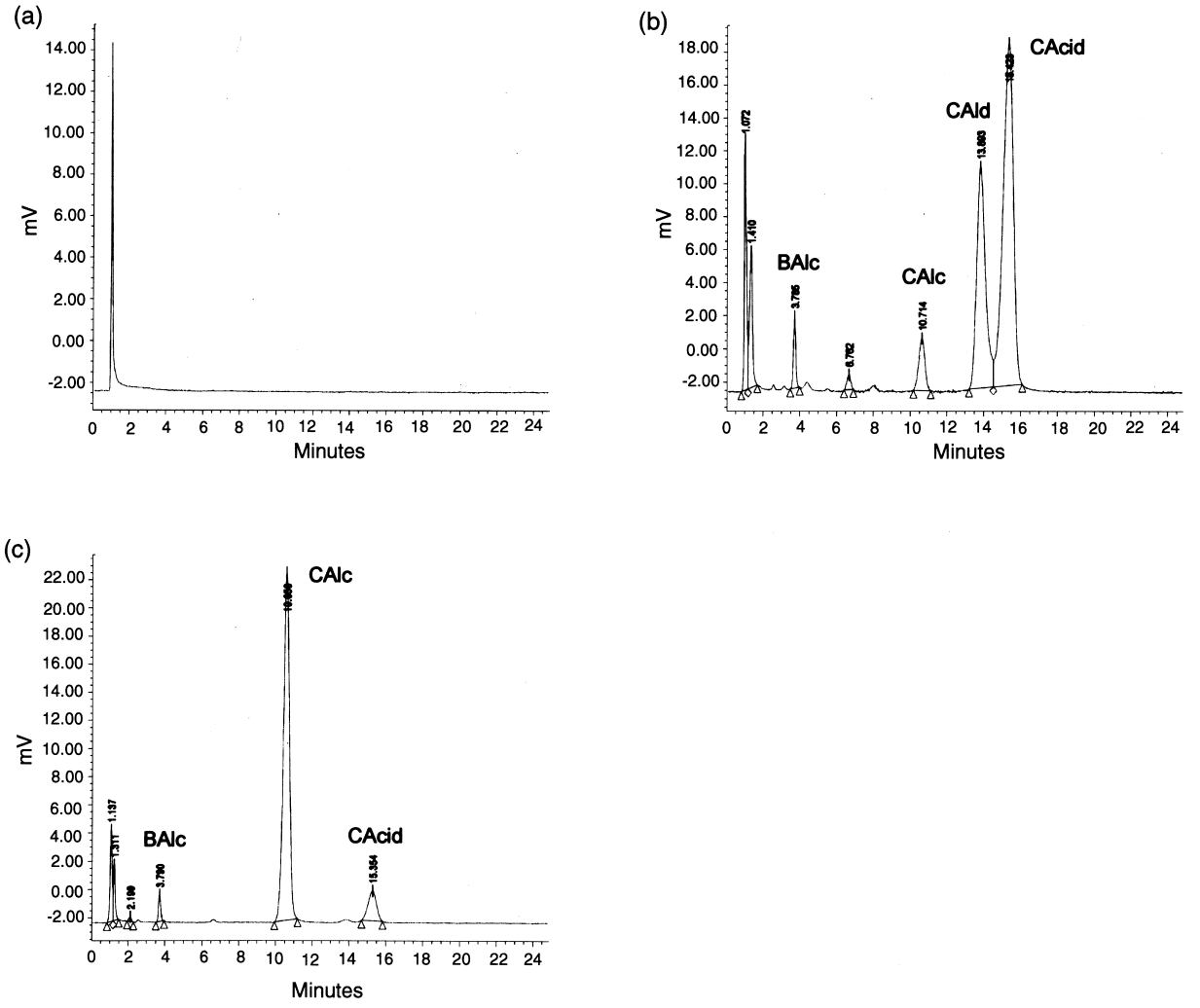

Fig. 3. Chromatographic analysis of cinnamic compounds extracted from human skin samples following application of cinnamaldehyde or cinnamic alcohol. Typical HPLC traces from analysis (UV detection of cinnamic compounds at 254 nm) of (a) sodium citrate–methanol buffer only; (b) supernatant following extraction of cinnamic compounds from skin homogenates generated from full-thickness skin topically applied with 78 μmol CAld and (c) 78 μmol CAlc.

0.17 to 2.52% for cinnamic alcohol, 0.24 to 9.14% for cinnamaldehyde and 0.26 to 6.43% for cinnamic acid. The mean RSDs (±SD) were 0.55±0.52, 2.41±2.59 and 1.56±1.78% for cinnamic alcohol, cinnamic aldehyde and cinnamic acid, respectively.

3.2.3. Inter-assay variation

The RSDs for cinnamic alcohol, cinnamaldehyde and cinnamic acid, respectively, as determined from $n=20$ HPLC runs, were 2.10, 4.16 and 2.26%.

3.2.4. Quantification of cinnamic compounds in receptor fluid

To illustrate the quality of data obtained from the methods described here, cumulative penetration profiles showing the total levels of penetrated cinnamic compounds (given as mean±SD of initial dose) following neat cinnamaldehyde (Fig. 5a) and cinnamic alcohol (Fig. 5b) are provided.

Following neat cinnamaldehyde application, a total of 9.5±1.6% of the initial cinnamaldehyde dose

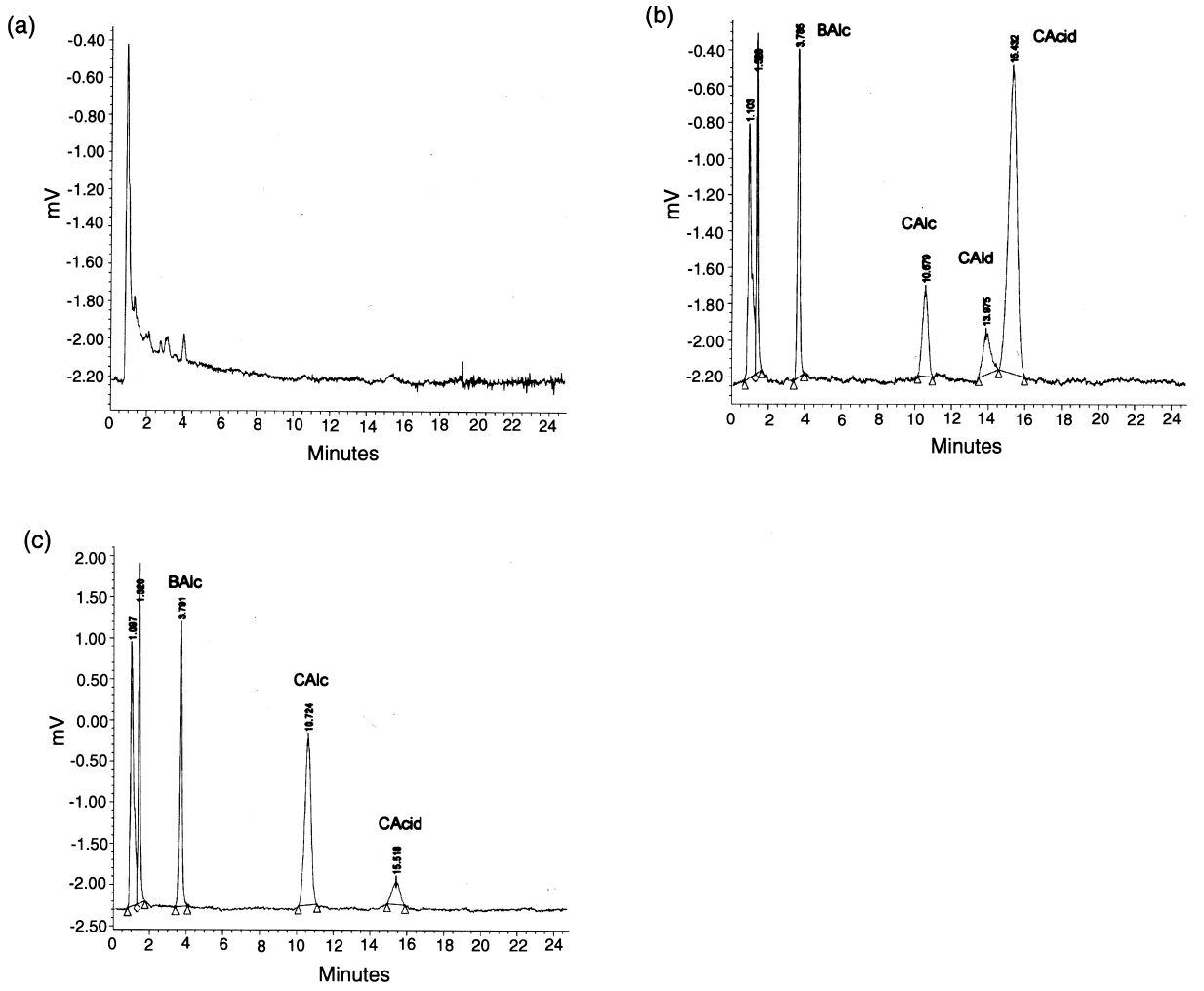

Fig. 4. Chromatographic analysis of extracted cinnamic compounds following treatment of cinnamaldehyde or cinnamic alcohol to human skin homogenates. Typical HPLC traces from analysis (UV detection of cinnamic compounds at 254 nm) of (a) potassium phosphate–methanol buffer only; (b) filtrate following extraction of cinnamic compounds from skin homogenates treated with 5 μmol CAld and (c) 5 μmol CAlc.

had penetrated the skin as some form of cinnamic compound (Fig. 5a). Individually, the cumulative penetration of parent cinnamaldehyde, cinnamic alcohol and cinnamic acid metabolites at 24 h reached levels of 2.6±1.0, 2.4±1.0 and 4.4±1.9%, respectively, and the levels of metabolites began to plateau at 22–24 h. At the end of the 2 h experiment, a total of 1.3±0.5% of the initial cinnamaldehyde dose had penetrated as either cinnamic alcohol or cinnamic acid metabolite (Fig. 5a inset).

Following 24 h human skin absorption of cinnamic alcohol, a total of 1.3±0.1% of the initial dose of cinnamic alcohol penetrated the skin as cinnamic alcohol and 0.6±0.1% had penetrated as cinnamic acid metabolite (Fig. 5b). No cinnamic alcohol-derived cinnamaldehyde metabolite was detected (limit of detection $<$0.08 nmol/20 μl analyte) in any receptor fluid samples following neat cinnamic alcohol application. At the end of the 2 h experiment, a total of 0.11±0.01% cinnamic alcohol and 0.08±0.01% cinnamic acid had penetrated the skin (Fig. 5b inset).

Table 3
Data from standard curve measurements: (a) mean data from $n=3$ sets of 10 standard solutions (1–10) as described in Table 2, (b) eight individual standard curve sets (A–H)

Std	Cinnamic alcohol (CAIc), $n=3$, Pkarea CAIc/BAIc±SD	Cinnamaldehyde (CAId), $n=3$, Pkarea CAIdIBAIc	Cinnamic acid (CAcid), $n=3$, Pkarea CAId/BAIc
(a)			
1	1.19±0.09	0.32±0.01	0.87±0.02
2	2.57±0.15	0.75±0.04	1.91±0.04
3	3.22±0.19	0.96±0.01	2.39±0.07
4	3.51±0.17	1.04±0.02	2.70±0.01
5	4.14±0.27	1.25±0.01	3.12±0.07
6	4.67±0.23	1.40±0.01	3.49±0.02
7	5.16±0.22	1.57±0.01	3.89±0.06
8	7.70±0.44	2.54±0.34	6.08±0.49
9	10.45±0.48	3.46±0.50	8.19±0.79
10	12.96±0.68	4.82±0.06	11.09±0.30
	$m=4.22\pm0.20$	$m=1.56\pm0.11$	$m=4.13\pm0.28$
	$c=-0.09\pm0.03$	$c=-0.30\pm0.08$	$c=-0.43\pm0.17$
	$r^2=0.999$	$r^2=0.993$	$r^2=0.996$

(b)	Cinnamic alcohol			Cinnamaldehyde			Cinnamic acid		
Std curve	m	c	r^2	m	c	r^2	m	c	r^2
A	5.262	−0.572	0.998	1.941	−0.388	0.999	4.506	−0.493	0.999
B	4.756	0.022	0.999	1.827	−0.157	0.999	4.048	−0.098	0.999
C	4.635	−0.034	0.999	1.748	−0.106	0.999	4.134	−0.150	0.999
D	4.767	0.075	0.999	1.715	0.114	0.999	4.207	0.222	0.999
E	5.779	−0.483	0.998	1.960	0.285	0.998	4.573	−0.422	0.998
F	5.541	0.319	0.999	1.567	−0.167	0.999	4.644	−0.373	0.999
G	5.139	−0.456	0.999	1.588	−0.211	0.997	4.391	−0.421	0.997
	5.328	−0.217	0.999	1.630	−0.245	0.999	4.102	−0.337	0.999
Mean	5.151	−0.048		1.747	−0.109		4.326	−0.259	
SD	0.407	0.360		0.152	0.213		0.232	0.238	

m = Gradient, c = intercept r^2 = correlation coefficient.

The total recoveries from the 24 h skin absorption and metabolism experiments for cinnamaldehyde and cinnamic alcohol are given in Table 4.

Fig. 6 illustrates the changes observed in cinnamic metabolite penetration into receptor fluid following preapplication of pyrazole to full-thickness skin in the skin absorption model system. In comparison to vehicle control samples, parent compound penetration was not significantly affected by the preapplication of pyrazole. The levels of both cinnamaldehyde-derived cinnamic alcohol and cinnamic acid metabolites were both reduced significantly ($P<0.05$) following 80 or 320 μmol pyrazole preapplication (Fig. 6a). Following cinnamic alcohol application, the level of cinnamic acid metabolite was significantly reduced only upon 320 μmol pyrazole preapplication (Fig. 6b).

3.2.5. Extraction efficiency of cinnamic compound isolation from skin homogenates

Extraction efficiencies for the individual recoveries of 10 μmol of cinnamaldehyde, cinnamic alcohol and cinnamic acid from human skin homogenates ($n=3$ for each) were calculated to be 88.3±2.8, 99.8±4.3 and 87.1±6.8%, respectively. In each case, a single peak for each extracted cinnamic compound was observed in the chromatograms indicating that no metabolism had taken place during

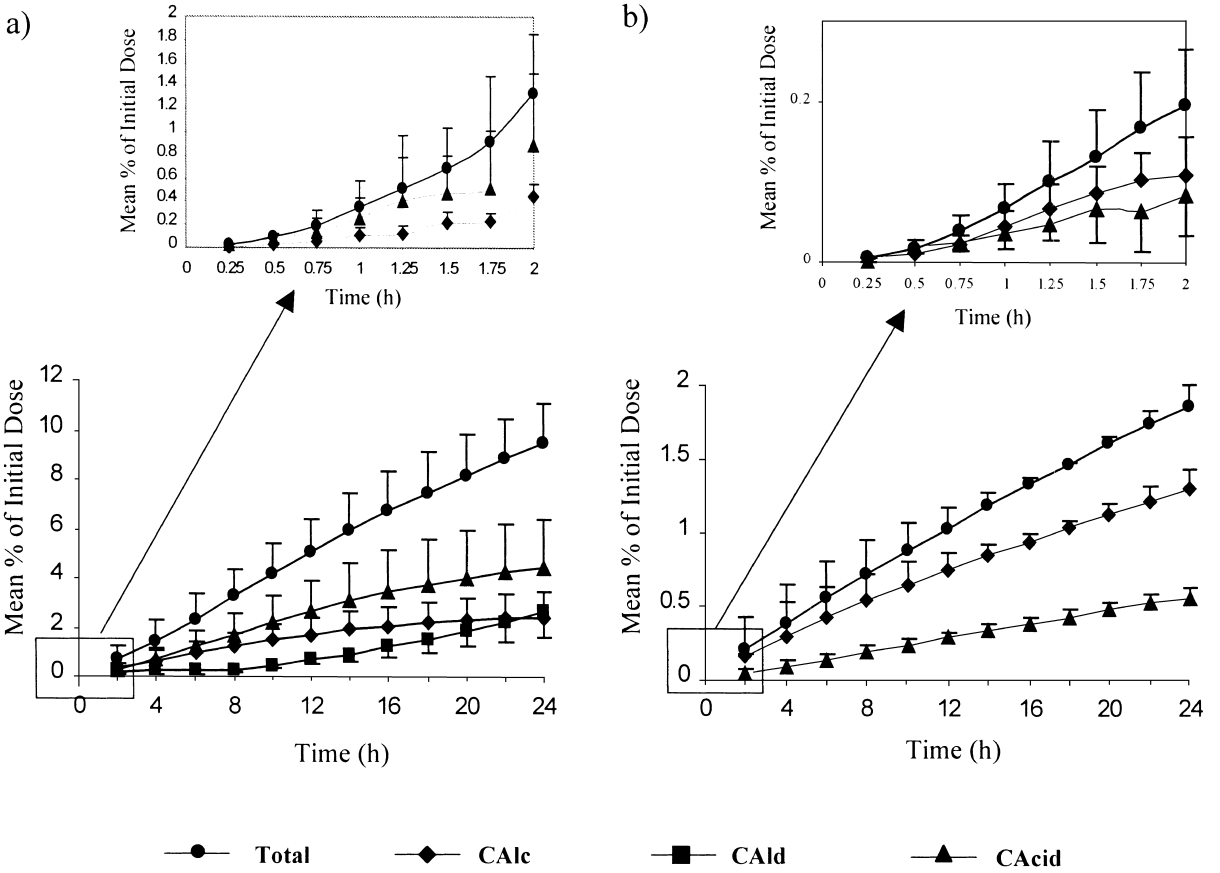

Fig. 5. Cumulative 24-h penetration profiles of (a) parent cinnamaldehyde and (b) parent cinnamic alcohol and their metabolites through human skin. Data are shown for cinnamic compound penetration through human skin following application of (a) 78 μmol neat cinnamaldehyde or (b) 78 μmol neat cinnamic alcohol. Values are given as mean±SD (%) of initial dose. The insets show the penetration profile of cinnamic compounds during the first 2 h after application.

Table 4
Total recovery data from skin absorption and metabolism experiments – recoveries (mean % initial dose±SD) from ($n=4$ for cinnamaldehyde and $n=3$ for cinnamic alcohol) experiments with full-thickness skin using the skin absorption model

24 h exposure (10 μmol neat compound)	Penetrated	Unabsorbed (average values)	Evaporated	Within skin	Total
Cinnamaldehyde applied					
Parent cinnamaldehyde	2.6±1.0	55.3	1±0.1	3.3±1.7	62.2
Cinnamic alcohol metabolite	2.4±1.0	0	0	0.4±0.2	2.8
Cinnamic acid metabolite	4.4±1.9	10.6	0	2.9±1.0	17.9
					=82.9*
Cinnamic alcohol applied					
Cinnamaldehyde metabolite	0	3.9	0.10±0.05	0	4.0
Parent cinnamic alcohol	1.3±0.1	55.2	0.40±0.02	3.1±0.1	60.0
Cinnamic acid metabolite	0.6±0.1	0	0	0.4±0.2	1.0
					=65.0*

*Cinnamaldehyde that may have remained bound to skin protein or glutathione cannot be accounted for using this method.

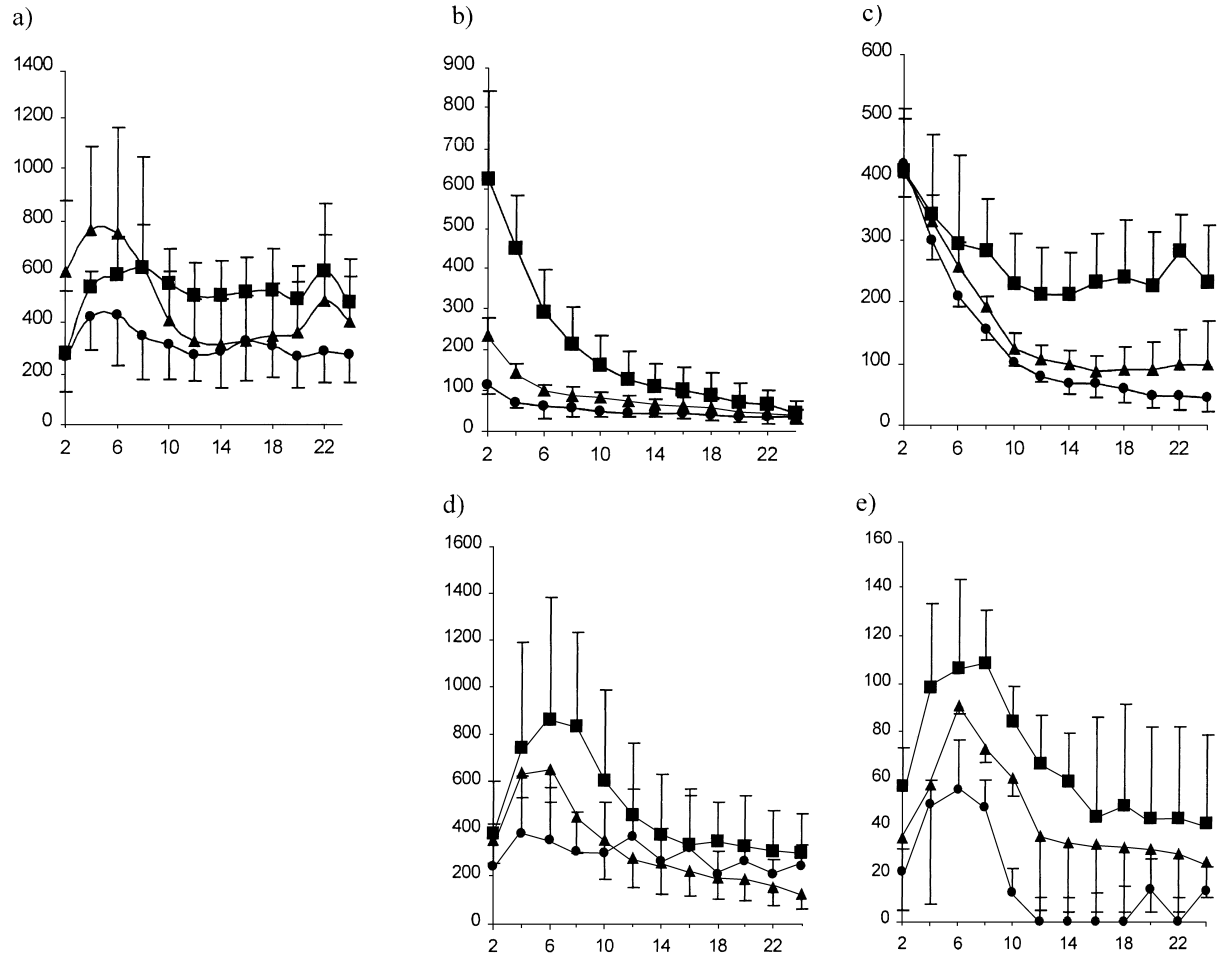

Fig. 6. Individual, cumulative 24-h penetration profiles of cinnamic compounds through human skin following preapplication of the skin with either vehicle (water) (coloured line), 80 (triangles) or 320 (circles) μmol aqueous pyrazole. Data are shown for cinnamic compound penetration through human skin following application of (a–c) cinnamaldehyde or (d–e) cinnamic alcohol. Values are given as mean±SD (%) of initial dose. Penetration of parent cinnamaldehyde is shown in (a) and reveals that in comparison to vehicle control there is no significant difference in penetration upon the preapplication of pyrazole inhibitor. Cinnamaldehyde-derived cinnamic alcohol and cinnamic acid metabolite penetration are shown in (b) and (c), respectively.

the extraction procedure using methanolic solutions. The corresponding values were applied to correct for extraction efficiency in calculating the levels of each cinnamic compound metabolite extracted from skin homogenates resulting from samples used in the skin absorption model experiments.

3.2.6. Chromatography of absorbed cinnamic compounds extracted from within full-thickness skin

Skin extracts (untreated control skin and cinnamic compound-treated skin) showed a consistent unidentified peak at a retention time of 1.30–1.41 min that was attributable to non-cinnamic-derived chemicals extracted from the skin (Fig. 3b and c). The peaks for cinnamic compounds and benzyl alcohol internal standard remained at the same retention times as seen in the other samples.

3.2.7. Quantification of absorbed cinnamic compounds extracted from within full-thickness skin

Following neat cinnamaldehyde application, parent cinnamaldehyde and cinnamic acid metabolite

could be isolated as free compounds from within the skin ($n=3$ samples) at statistically equivalent levels (2.9±1.0 and 3.3±1.7%, respectively). The level of free cinnamic alcohol metabolite was much lower (0.4±0.2%). In contrast, the predominant cinnamic compound extracted from within the skin after cinnamic alcohol application was parent cinnamic alcohol (3.1±0.1%). A low level of free cinnamic alcohol-derived cinnamic acid metabolite (0.4± 0.2%) could also be extracted from the skin homogenates. However, no cinnamaldehyde metabolite was detected in skin homogenate extracts following cinnamic alcohol application (Table 4).

3.2.8. Chromatography of absorbed cinnamic compounds extracted from treated skin homogenates

Similarly to the skin extracts from treated full thickness skin, the filtrate from the treated skin homogenate extractions showed a consistent unidentified peak at a retention time of 1.52–1.54 min that was attributable to non-cinnamic-derived chemicals extracted from the skin (Fig. 4b and c). Again the peaks for cinnamic compounds and benzyl alcohol internal standard remained at the same retention times as seen in the other samples.

3.2.9. Quantification of cinnamic compounds extracted from treated skin homogenates

To assess the extent of metabolism of cinnamaldehyde and cinnamic alcohol in the absence of the skin barrier properties, skin homogenates were treated with the compounds, and a new method used to extract cinnamic compounds. The extraction efficiencies of the method at pH 5 for each individual cinnamic alcohol, cinnamaldehyde and cinnamic acid were 89.6±15.0, 101.6±2.7, 87.8±8.1%, respectively. Data for cinnamic compound recoveries (μmol/mg protein) from skin homogenates that had been incubated with either cinnamaldehyde or cinnamic alcohol for 30 and 90 min are presented in Table 5. Total recovery data, given as total mean % of initial dose±SD recovered, are also given in Table 5.

4. Discussion

The data presented within this study shows that a reproducible HPLC assay has been developed for the simultaneous analysis and statistical quantification of nmol of non-radiolabelled cinnamaldehyde, cinnamic alcohol and cinnamic acid. The major benefit of this method, when used in conjunction with the skin absorption model, is that aqueous physiological

Table 5
Total recovery data from skin absorption and metabolism experiments – recoveries of individual cinnamic compounds (mean μmol/mg protein±SD) and total recovery (mean % of initial dose±SD) from experiments with skin homogenates ($n=3$ humans) treated with cinnamic compounds

Sample	Applied dose (2.06±0.39 μmol/mg protein)	CAlc (μmol/mg protein)	CAld (μmol/mg protein)	CAcid (μmol/mg protein)	Total recovery (%)
Incubation time 30 min					
Control (100°C)	Cinnamaldehyde	0	1.67±3.86	0.29[a]	84.4±11.4
	Cinnamaldehyde	0.28±0.06	0.49±0.40	1.01±0.49	91.0±9.9
Control (100°C)	Cinnamic alcohol	1.56±1.84	0	0	85.2±14.9
	Cinnamic alcohol	1.42±1.60	0	0.16±0.08	78.8±18.6
Incubation time 90 mm					
Control (100°C)	Cinnamaldehyde	0	1.74±0.24	0.29[a]	89.1±3.5
	Cinnamaldehyde	0.41±0.04	0.40±0.26[b]	0.99±0.35	85.8±9.7
Control (100°C)	Cinnamicalcohol	1.84±0.14	0.01[a]	0	97.2±4.8
	Cinnamic alcohol	1.62±0.28	0	0.14±0.07	86.3±13.0

[a] Compound detected in one sample only.
[b] Compound detected in two samples only.

buffer (pH 7.4) and methanolic samples generated in the flow-through skin absorption model system, can be analysed directly without the use of a multi-step solvent extraction procedure. However, this HPLC method of quantification could also be useful for analysing cinnamic compounds generated in other types of experiments, for example, those isolated from cell culture media.

As verified by UV spectroscopic analysis, the major λ_{max} value was 304 nm for cinnamic alcohol, cinnamaldehyde and cinnamic acid. However, linearity in generating standard curves could not be achieved at this wavelength. A secondary peak was also observed with λ_{max} 245 nm for cinnamic alcohol and cinnamaldehyde, and 254 nm for cinnamic acid. Linear calibration data could be generated for absorbances up to 0.1 and for concentrations up to a maximum of 1 μmolar, using this secondary wavelength. Pyrazole did not absorb UV radiation at 254 nm and hence, did not interfere with cinnamic compound analysis.

Given the consistent nature of the calibration curves generated over a period of 18 months, it was possible to use equations that were derived from mean values of triplicate measurements for multiple analyses. In doing this, the same calibration curve should not be used for a period longer than 2–3 months and all samples must be diluted to yield PkareaCC/PkareaBAlc ratios of <8 for cinnamic alcohol, <3 for cinnamaldehyde and <8 for cinnamic acid for maximum accuracy of concentration determination. Best practice however, as performed in runs A–H, is to run the set of standard solutions at the beginning of each sample run, so that each set of samples has it's own related 10-point calibration curve.

A noteworthy observation, is that the very first injection, following storage of the column for more than a couple of days in 100% methanol, always led to a superior baseline resolution for the cinnamaldehyde peak. This yielded a slightly but significantly higher peak area than was seen during routine ongoing sample analysis for cinnamaldehyde. Hence, when this occurred, the RSD between the first standard and subsequent standards within a run was $>10\%$ for cinnamaldehyde. It is recommended that following column storage, a minimum of three equivalent standards should be run at the beginning of a new run and data from the first two of these standards should not be used. Also, of paramount importance to the reproducibility of the calibration curves, is a high level of precision in the measurement of cinnamic compounds in standard solutions and accurate pipetting of volatile methanolic solutions during the dilution steps in preparing standards. A correlation coefficient of $>0.99\%$ for the calibration curve should always be achieved.

It has also been shown that our method could be successfully applied to the investigation of cinnamaldehyde and cinnamic alcohol metabolism in full-thickness skin [18] and skin homogenates. Protein-reactive cinnamaldehyde was detoxified to cinnamic alcohol and cinnamic acid in both full-thickness skin absorption and metabolism experiments and in skin homogenates. Conversion of cinnamic alcohol to protein-reactive cinnamaldehyde (which is hypothesised to act as the hapten in cinnamic alcohol sensitisation) was only observed on the surface of full-thickness skin. This may indicate that air-oxidation of the alcohol to the aldehyde or that skin microfloral metabolism may play roles in the generation of hapten. However, cinnamic alcohol-derived cinnamic acid, presumably generated via a cinnamaldehyde intermediate, was also observed in both systems. It is possible that the levels of cinnamaldehyde intermediate generated are too low to be detected by our method or that as soon as conversion to cinnamaldehyde takes place, it is rapidly converted to cinnamic acid, or it may bind to glutathione or skin proteins and remain undetected.

Significant reductions in the levels of penetrated (cinnamic alcohol and cinnamic acid) metabolites (but not parent compound) upon preapplication of the ADH inhibitor pyrazole to the skin, suggest that we have observed changes in genuine skin metabolism of these compounds [18]. The levels of cinnamic metabolites generated in receptor fluid following skin absorption were typically low (nmol–μmol). Our method offers significant improvements upon previously published methods [18,20,23], for the quantification of small changes in levels of cinnamic metabolites generated in biological systems. The absolute quantification of cinnamic compounds, performed in relation to carefully constructed calibration curves (where $r^2>0.99$), is crucial for such comparisons to be made.

The analysis of cinnamic compounds from within the skin is more complex than analysing compounds that have penetrated through, evaporated from or remained unabsorbed on the skin. However, a relatively simple and high efficiency (>87% recovery) single solvent method has now been developed for direct cinnamic compound extraction from both full-thickness human skin and treated skin homogenates, into a methanolic solution. It is expected that our extraction method could also be used for extracting cinnamic compounds from within the skin taken from other species (for example, rat, mouse, guinea-pig) and for other similar methanol-soluble compounds (such as benzaldehyde and relatives) for direct HPLC analyses. This single solvent extraction method, together with the specifically developed HPLC assay, now enables further investigation and quantification of skin absorption and metabolism of cinnamic compounds to be performed.

Acknowledgements

We gratefully acknowledge the support of the European Cosmetic, Toiletry and Perfumery Association (COLIPA) and the Research Institute for Fragrance Materials (RIFM).

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