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Liquid chromatographic assay for the simultaneous determination of indole-3-carbinol and its acid condensation products in plasma

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Abstract

A high-performance liquid chromatographic method was developed for the simultaneous determination of indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane (LTr_1), and indolo[3,2b]carbazole (ICZ). Compounds were extracted from mouse plasma using *tert*.-butyl methyl ether, incorporating 4-methoxy-indole as internal standard. Chromatographic separation utilized a Waters Symmetry RP18 in tandem with a Thermoquest BDS C_{18} column, an acetonitrile–water gradient and UV (280 nm) in series with fluorescence (ex. 335 nm; em. 415 nm) detection. Calibration curves were linear (r^2>0.99) between 50 and 15,000 ng/ml for I3C; 150 and 15,000 ng/ml for LTr_1; and 0.15 and 37.5 ng/ml for ICZ and the method was reproducible and precise (within-day and between-day coefficients of variation below 9.7 and 13%, respectively). The method described is suitable for comprehensive pharmacokinetic studies with indole-3-carbinol.

Keywords: Indole-3-carbinol; 3,3′-Diindolylmethane

1. Introduction

Indole-3-carbinol (I3C) is a dietary constituent derived from indolyl-methyl glucosinolate, found in cruciferous vegetables such as cabbage, broccoli and Brussels sprouts. Upon maceration, endogenous enzymes, particularly myrosinase, lead to the breakdown of indolyl-methyl glucosinolate to form a range of indoles, including I3C [1]. I3C has potential as a chemopreventive agent: it has been shown to have the ability to block tumor initiation [2], with a major mechanism of action being its capacity to induce both phase I and II enzymes involved in both carcinogen [2,3] and estrogen [4] metabolism. Clinically, I3C has been shown to increase the 2-hydroxyestrone to 16α-hydroxyestrone ratio in women, promising efficacy in the prevention of hormonal tumors, including breast and cervical cancer [5,6]. There is also evidence to suggest that I3C can

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suppress tumor formation by modulating cell-signaling pathways, resulting in inhibition of cell proliferation and induction of apoptosis [7] as well as having the ability to act as a free-radical scavenger [8].

Following oral administration to animal species, I3C undergoes acid-catalyzed condensation in the stomach, which also occurs under acidic conditions in vitro (0.05 *M* hydrochloric acid, pH 1.5 for 60 min), and at least 15 oligomeric products are formed [9]. 3,3′-Diindolylmethane (DIM) and [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane (linear trimer; LTr_1) (Figs. 1 and 2) are two of the major products found under both in vitro and in vivo conditions [9,10,14,18]. These compounds have been shown, using in vitro studies, to bind to the aryl hydrocarbon-receptor (AhR) [11,12], modulating estrogen receptor function [12,13], and to inhibit proliferation of human breast tumor cells in culture [13], a finding confirmed in our laboratory (unpublished data). Another oligomer generated following acid-treatment of I3C, 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b′:7,8-b″]tri-indole (cyclic trimer; CTr) (Fig. 1), has been found in plasma and liver of rats following oral dosing with I3C [14,18]. However, CTr has not been identified in all in vivo studies [9] and it did not inhibit breast tumor cell growth, in vitro, showing a low binding affinity to the AhR and failure to activate AhR-mediated pathways [15]. Indolo[3,2b]carbazole (ICZ; Fig. 1) is formed in negligible amounts from acid condensation of I3C in vitro (Fig. 2) and reportedly in small amounts in vivo following I3C administration to rodents and humans [16]. However, it was found in our laboratory (unpublished data) that ICZ inhibits proliferation of breast tumor cells in culture and it has been shown to bind strongly to the AhR [14,17], thus regulating expression of CYP 1A1, and consequentially estradiol metabolism. ICZ also appears to cause ''cross-talk'' between the AhR and estrogen receptor-mediated signal transduction pathways by a mechanism independent of CYP-induced hormone metabolism [17]. A second linear trimer (LTr_2) (Fig. 1) has been found in acid mixture, in vitro [9,10] (Fig. 2), but it has not been identified in plasma or tissues following I3C administration to animals.

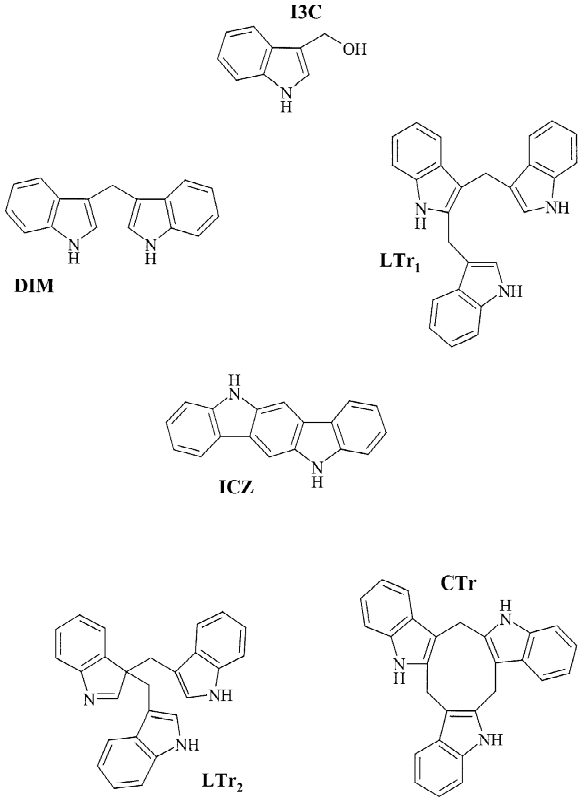

Fig. 1. Structure of indole-3-carbinol and its major acid condensation products. I3C, indole-3-carbinol; DIM, 3,3′-diindolylmethane; LTr_1, (1st) linear trimer; ICZ, indole[3,2b]carbazole; LTr_2, (2nd) linear trimer; CTr, cyclic trimer.

A number of disposition studies with I3C have been carried out [8,9,18,19], in most cases involving radiolabelled I3C [8,18,19] and lacking emphasis on simultaneous quantification of individual acid condensation products [8,19]. I3C-derived species have been measured in the gut, liver, urine and feces as well as in plasma [8,9,18–20]. Plasma levels of total I3C-derived species were found to reach a maximum 30 min after administration of ^{14}C labeled I3C [8]. Except for one recent study in humans, in which DIM plasma concentrations were quantified following I3C administration, and the pharmacokinetics of this compound assessed [21], comprehensive pharmacokinetic, metabolism and disposition studies of I3C have generally not been carried out, even when quantification of individual products should have been possible.

Despite the prior utilization of high-performance liquid chromatographic (HPLC) methodology for the

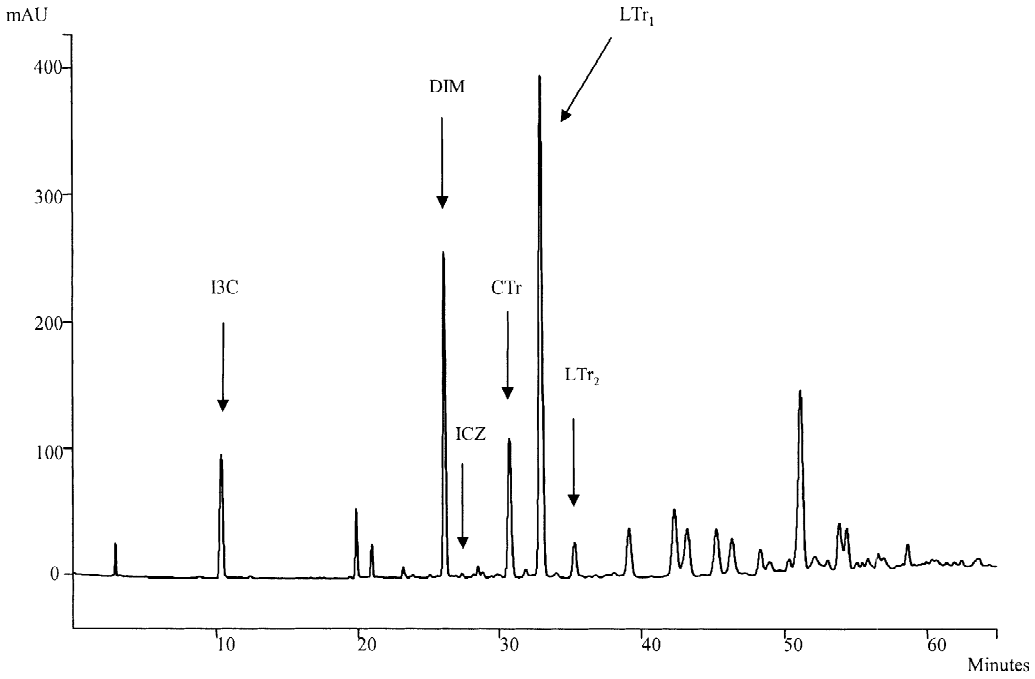

Fig. 2. HPLC chromatogram resulting from the analysis of species formed from I3C after 10 min in an acidic aqueous (pH 1) environment. The column was eluted at 1 ml/min using an acentonitrile–water gradient as described in Section 2.4. UV detection was at 280 nm.

assessment of I3C and its oligomers [9,18,19,21], this is the first fully validated assay to be reported, capable of concise determination of all I3C-derived species of major interest. The chromatographic method described in this paper is the first to enable simultaneous separation and quantification of the parent compound, I3C, alongside its acid condensation products of major interest, and subsequent to further validation, the assay is likely to be suitable for use in species other than the mouse, allowing comprehensive pharmacokinetic analyses in animals and humans.

2. Experimental

2.1. Chemicals and reagents

I3C and 4-methoxy-indole were purchased from Sigma–Aldrich (Poole, Dorset, UK). DIM was purchased from LKT Laboratories (St. Paul, MN, USA). ICZ and LTr_1 were synthesized in this laboratory to 97% purity according to previously described methods [9,22] (see Section 2.2).

Acetonitrile and *tert*.-butyl methyl ether (both HPLC grade) and HEPES sodium salt were purchased from Sigma–Aldrich. Ammonia solution, dichloromethane, DMSO, ethanol, methanol, hydrochloric acid, petroleum ether 60–80%, sulfuric acid and tetrahydrofuran were of HPLC or analytical grade and were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Quinoline and triethylorthoformate were of the highest grade available and were also obtained from Fisher Scientific. HEPES sodium salt was purchased from Sigma–Aldrich.

Water was prepared by an ultrapure water system (ELGA, Bucks, UK) and sonicated for 1 min prior to use.

Standard mouse plasma was obtained from Charles River Laboratories (Margate, Kent, UK).

2.1.1. Animals

Female CD-1 mice (25–30 g) obtained from Harlan UK (Bicester, UK) were housed in Moredun

Isolators (Moredun Animal Health, Edinburgh, UK) under negative pressure with a 12-h light–dark cycle (temperature range, 20–23 °C; humidity, 40–60%) and were fed food and water freely. The animals were allowed to acclimatize for 1 week before initiation of preliminary pharmacokinetic studies (Section 2.13).

2.2. Synthesis of standards

2.2.1. ICZ

Synthesis of ICZ was according to the method previously described [22]. In brief, concentrated sulfuric acid (three drops) was added to DIM (1.23 g, 0.02 mol) and triethyl orthoformate (0.82 ml, 0.02 mol) in absolute (dry) methanol (30 ml) at room temperature. The mixture was heated for 5 min under reflux. The precipitated crystals were collected and, after careful drying, were recrystallized from quinoline. ICZ was formed as light yellow crystals and, on HPLC analysis (see Section 2.4), exhibited one major peak. ICZ was characterized using mass spectrometry (see Section 2.12) and had a purity of 97–98%.

2.2.2. LTr_1

Synthesis of LTr_1 was according to a method adapted from De Kruif et al. [9]. I3C (2 g) was dissolved in DMSO (8 ml) and subsequently added to hydrochloric acid (2 l, 0.05 *M*). The mixture was stirred for 10 min, after which the resulting precipitate of I3C and its oligomers was removed, washed with water and extracted into dichloromethane (50 ml). After evaporation of the dichloromethane, the acid condensation products were dissolved in ethanol (50 ml) and precipitated by the drop-wise addition of water (100 ml). The precipitate was collected and dried under vacuum in a desiccator.

LTr_1 was initially separated from other acid condensation products using flash chromatography. The reaction mixture, dissolved in tetrahydrofuran–petroleum ether 60–80% (25:75, v/v), was added to a short silica column (5×9 cm). LTr_1 was separated using tetrahydrofuran–petroleum ether (25:75, v/v) as the mobile phase. Fractions were collected and compared to a standard, obtained from a previous small-scale preparation using the same method, using silica thin layer chromatography. Fractions containing LTr_1 were evaporated to dryness, dissolved in ethanol (10 ml) and precipitated with water (drop-wise; 10 ml). In the second purification step, the mixture was purified by preparative HPLC. The chromatographic system consisted of a Gilson 306 pump, Gilson 811C Dynamic mixer (Middleton, WI, USA), Rainin Dynamax UV-1 detector set at 280 nm (Varian, Surrey, UK), Rheodyne Model 7125 injection valve with a 500-μl sample loop (Anachem, Luton, Bedfordshire, UK) and a Thermoquest C_{18} BDS (250×21.2 mm, 5 μm) column (Runcorn, Cheshire, UK). The reaction mixture (10 mg) was dissolved in acetonitrile (500 μl) and oligomers were eluted using acetonitrile–water (60:40, v/v) from 0 to 33 min, followed by a step up to 100% acetonitrile over the next 2 min and then 100% acetonitrile isocratically from 35 to 52 min using a flow-rate of 10 ml per min. Subsequently, the column was re-equilibrated at initial conditions. LTr_1 fractions were collected (28 min), and acetonitrile was removed using a rotary evaporator. LTr_1 was extracted into dichloromethane, washed with water and evaporated to dryness. LTr_1 was dissolved in ethanol, precipitated with water, centrifuged and dried under vacuum in a desiccator, yielding LTr_1 as a white powder, the identity of which was confirmed by mass spectrometry (see Section 2.12). On HPLC analysis (see Section 2.4), LTr_1 exhibited one major peak and had a purity of 98–99%.

2.3. Small scale synthesis of I3C acid condensation products for analysis and identification

I3C acid condensation products were prepared according to a slight modification of a previously described method [10]. Briefly, hydrochloric acid (28 μl, 1 *M*) was added to an aqueous solution of I3C (250 μl, 12 m*M*). The mixture was vortexed for 10 min and neutralized with aqueous ammonia (90 μl, 0.5 *M*). The resulting green precipitate was dissolved in tetrahydrofuran (670 μl) and diluted 1:20 in acetonitrile–50 m*M* HEPES buffer, pH 7.4 (40:60, v/v), before analysis by HPLC. Following separation of all acid condensation products using the HPLC conditions described in Section 2.4, individual fractions were collected and each oligomer characterized by mass spectrometry (see Section 2.12).

2.4. HPLC apparatus

The Varian Prostar HPLC system consisted of a 230 pump, a 410 autosampler, a 310 UV detector and a 363 fluorescence detector. The compounds were separated at room temperature on a Waters Symmetry RP18 (4.6 mm×50 mm, 5 μm) column (Hertford, UK) in tandem with a Thermoquest BDS C_{18} (250 mm×4.6 mm, 5 μm) column with a Phenomenex C_{18} ODS (4 mm×3 mm) guard column (Cheshire, UK). The compounds were quantified by UV detection at 280 nm, with the exception of ICZ, which was quantified using fluorescence detection at 335/415 nm (ex./em.). The mobile phase consisted of acetonitrile–water and solutes were eluted using a flow-rate of 1.0 ml/min and a steady gradient program starting at 15% acetonitrile at 0 time, taken to 60% acetonitrile over the next 20 min, rising to 65% acetonitrile from 20 to 40 min and reaching 85% acetonitrile at the final time of 65 min. The column was re-equilibrated at initial conditions for 8 min before the next analysis.

2.5. Sample preparation

To mouse plasma (250 μl) in a screw cap centrifuge tube (4 ml) was added 4-methoxy-indole (2.5 μl of 0.4 mg/ml) as internal standard (I.S.). The samples were mixed and equilibrated at room temperature for 30 min prior to extraction twice with *tert*.-butyl methyl ether (750 μl) involving 2 min vortexing on each occasion. Subsequent to each extraction, the organic and aqueous layers were separated by centrifugation (2800 *g*, 10 min) and the organic (top) layers transferred to a fresh 4-ml tube. For each sample, the combined *tert*.-butyl methyl ether layers were evaporated rapidly under nitrogen in the presence of DMSO (15 μl). The extracted sample in DMSO was then diluted using acetonitrile–50 m*M* HEPES buffer, pH 7.4 (40:60, v/v; 135 μl). Fifty μl of this diluted sample were subsequently injected into the HPLC system.

2.6. Standard solutions

Stock solutions of each compound were separately prepared in DMSO. I3C, DIM and LTr_1 were prepared at a concentration of 4 mg/ml, 4-methoxy-indole at 0.4 mg/ml and ICZ at 0.08 mg/ml. Stock solutions were stored at −80 °C. Preliminary experiments showed that all solutions were stable for a minimum of 6 months under these conditions.

2.7. Stability

In order to study the stability of compounds in plasma at a variety of possible conditions, blank (initially drug free) mouse plasma was spiked separately with a known amount of each stock solution to yield final concentrations of both 500 and 12,500 ng/ml for each of I3C, DIM, and LTr_1, and 1.5 and 37.5 ng/ml for ICZ. Plasma samples were then left at room temperature for 24 h; −20 °C for 30 days and then after three freeze–thaw cycles at this temperature; and −80 °C for 90 days. For each concentration under each condition, four samples were extracted and analyzed. The concentration of each compound after storage was related to the initial concentration as determined for samples that were freshly prepared and processed immediately.

To determine stability of extracted samples in the autosampler, quality control solutions were prepared in acetonitrile–50 m*M* HEPES, pH 7.4 (40:60, v/v), to yield concentrations of 500 and 12,500 ng/ml for I3C, DIM, and LTr_1, and 1.5 and 37.5 ng/ml for ICZ. Again, for each concentration, four samples were analyzed and the concentration for each compound after storage in the autosampler for 48 h was related to the initial concentration of samples analyzed immediately.

2.8. Calibration curves

Calibration samples were produced by spiking blank mouse plasma with a known amount of stock solution. Calibration curves were constructed using six concentrations of analyte in the range of 50–15,000 ng/ml for I3C and DIM; 150–15,000 ng/ml for LTr_1; and 0.15–37.5 ng/ml for ICZ. Curves were generated for each compound by plotting the peak area ratio (PAR) of the analyte and I.S. (PAR= analyte peak area/I.S. peak area) against theoretical concentrations. For ICZ, curves were additionally generated by simply plotting peak area (PA) against theoretical concentrations. Linear regression analysis (n=6) was used to determine correlation coefficients

(r^2 values) as well as the equations describing each analyte curve.

2.9. Quality control samples

The quality control samples (QCs) used for the validation consisted of the lower limit of quantification (LLOQ), a low quality control (LoQC), a medium quality control (MeQC) and a high quality control (HiQC), produced by spiking blank mouse plasma with four chosen concentrations of stock solution. The LLOQ, LoQC, MeQC and HiQC sample concentrations were equal to 50, 75, 7500 and 12,500 ng/ml, respectively, for I3C and DIM; 150, 200, 7500 and 12,500 ng/ml, respectively, for LTr_1; and 0.15, 0.225, 22.5 and 37.5 ng/ml, respectively, for ICZ. Intra-day validation studies ($n=6$ samples) and inter-day validation studies ($n=6$ days) were conducted using these QCs.

2.10. Recovery

The recovery of each indole from mouse plasma was assessed by comparison of the slope of a calibration curve of six concentrations, 50–15,000 ng/ml for I3C and DIM; 150–15,000 ng/ml for LTr_1; and 0.15–37.5 ng/ml for ICZ. Solutions were prepared so that they contained DMSO (15 μl) and acetonitrile–50 m*M* HEPES buffer, pH 7.4 (40:60, v/v) (135 μl) and compared to a calibration curve of the same concentrations extracted from plasma.

2.11. Limits of detection and quantification

The limit of detection (LOD), defined as three times the signal-to-noise ratio, was determined by injecting extracted samples of I3C and its oligomers. The lower limit of quantification (LLOQ) was established for each analyte according to the lowest concentration at which the coefficient of variation (C.V.) value was less than 15%. The LLOQ was set as the lowest concentration of the calibration curve.

2.12. Identification of I3C condensation products by mass spectrometry

Electron ionization (EI) spectra of I3C and its oligomers were obtained via direct insertion probe technique using a VG 70-SEQ mass spectrometer (Micromass, Manchester, UK). The conditions employed were 70 eV electron energy, 200 μA trap current and a source temperature of 250 °C.

2.13. Application of the method

The preliminary pharmacokinetic study described here was approved by the University of Leicester Ethical Committee for Animal Experimentation. This study involved a total of 18 mice and blood samples were collected, in triplicate, prior to dosing with I3C and then at 15 min, 30 min, 60 min, 120 min and 360 min following a single oral dose of I3C (250 mg/kg) suspended in corn oil. Blood was collected by cardiac exsanguination under halothane anesthesia and collected in heparinised tubes. The plasma fraction was immediately separated by centrifugation (5 min, 10,000 *g*) and stored at −80 °C until analysis. I3C and its condensation products were extracted and analyzed as described in Sections 2.4 and 2.5.

3. Results and discussion

The column combination chosen for this analysis resulted in a good separation of I3C and its oligomers. Using the Thermoquest BDS C_{18} column alone, separation of most products of the acid catalyzed condensation of I3C could be achieved. However, DIM and ICZ threatened to co-elute and since ICZ concentrations are predicted to be small this was not satisfactory. The addition of a 50 mm Waters Symmetry RP18 column, in series with the Thermoquest BDS column allowed good separation of the two compounds without adversely affecting the separation of the rest of the oligomers (Fig. 2). Under the final HPLC conditions, the first peak to elute was I3C, which had a k' value of 2.70, and baseline separation of all compounds was achieved: $\alpha_{\text{I3C-I.S.}}=2.04$; $\alpha_{\text{I.S.-DIM}}=1.46$, $\alpha_{\text{DIM-ICZ}}=1.08$; $\alpha_{\text{ICZ-LTr}_1}=1.18$.

Fractions were collected following injection of I3C acid reaction mixture onto the HPLC (Section 2.4), and subsequent mass spectral analysis yielded a set of spectra that were analyzed and subsequently compared to spectra reported in a previous publication in order to aid and confirm compound identifi-

cation [10]. Peaks characterized as DIM, LTr_1, LTr_2 (a second linear trimer, Fig. 1) and CTr gave spectra that were almost identical to those previously reported. In the current study, separated peaks that eluted after 36 min were identified as indoles but they could not be definitively characterized, due either to the existence of a number of structural isomers or cross-contamination of fractions. Additionally, the identification of DIM and ICZ was confirmed by comparison of HPLC retention times with those produced from authentic standards.

The current method was validated for I3C, DIM, LTr_1 and ICZ. We found DIM and LTr_1 to be the products formed in highest quantity following exposure of I3C to acidic conditions (Fig. 2). These indoles are also the major products reportedly found in vivo [9,14,18,21]. ICZ is a minor acid condensation product of I3C but it is of great interest due to its anti-estrogenic and anti-proliferative potential [14,17].

The stability of the indoles studied was of particular importance. It is acknowledged that I3C is unstable under acidic conditions but there have also been some reports of instability under neutral pH conditions, where I3C is apparently converted to DIM [4,23]. However, in the current study, I3C was found to be completely stable at neutral pH under a range of conditions. Thus, I3C, as well as DIM, LTr_1, ICZ and I.S. (4-methoxy-indole) were found to be completely stable under all conditions tested (Section 2.7), such that the mean recovery ($n=4$ for each compound concentration under each condition) was found to be 100% with excellent reproducibility ($\leq$9% variation for each sample). A number of groups have used ethyl acetate for indole extraction from plasma [14,18,19] but, in our hands, ethyl acetate facilitated the conversion of I3C to DIM. This conversion is possibly caused by minor amounts of acetic acid, formed by hydrolysis of ethyl acetate, and may be a function of solvent storage time. The use of *tert.*-butyl methyl ether avoided I3C instability and a small amount of DMSO (15 μl) added to extractions prior to drying down under nitrogen flow completely avoided I3C breakdown during this process.

Chromatograms from the analysis of I3C, DIM, LTr_1 and ICZ standards, extracted from blank plasma, are depicted in Figs. 3 and 4. Calibration curves for I3C, DIM, LTr_1 and ICZ were linear within the wide range investigated (50–15,000 ng/ml for I3C and DIM; 150–15,000 ng/ml for LTr_1; and 0.15–37.5 ng/ml for ICZ). The standard curves were designed to address the anticipated concentrations encountered in rodent plasma following oral and intravenous dosing with I3C and DIM at doses believed to be of therapeutic advantage (250 mg/

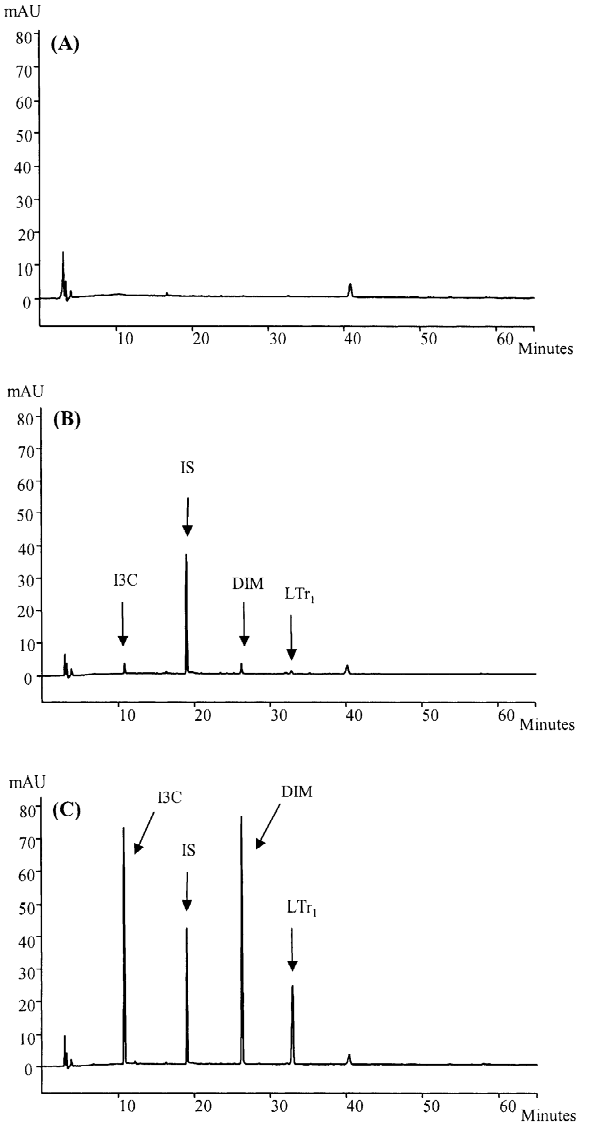

Fig. 3. HPLC chromatograms from the analysis of standard mixtures of I3C and its acid condensation products using UV detection at 280 nm. (A) Blank plasma, (B) 150 ng/ml standard mixture, (C) 5000 ng/ml standard mixture. I3C, indole-3-carbinol; DIM, 3,3′-diindolylmethane; LTr_1, 1st linear trimer; IS, internal standard.

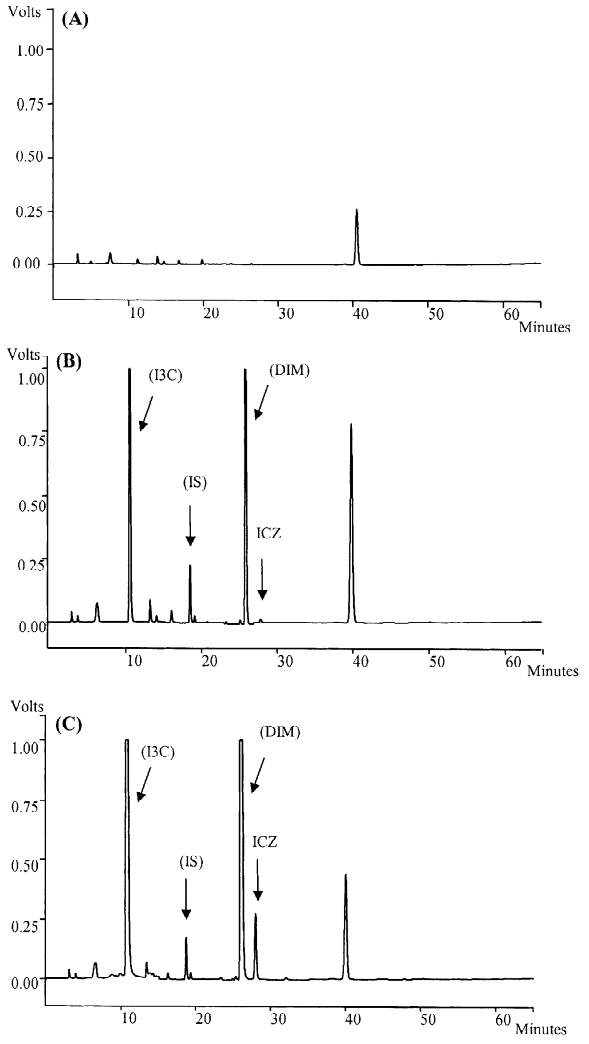

Fig. 4. HPLC chromatograms from the analysis of ICZ using fluorescence detection; 335/415 nm (ex./em.). Samples were spiked with a standard mixture containing ICZ, I3C, DIM, I.S., and LTr_1, although ICZ only was quantified using fluorescence detection and LTr_1 did not fluoresce at the set excitation and emission wavelengths. (A) Blank plasma, (B) 0.15 ng/ml ICZ, (C) 10 ng/ml ICZ. ICZ, indolo[3,2b]carbazole; I3C, indole-3-carbinol; DIM, 3,3′-diindolymethane; IS, internal standard.

kg). Correlation coefficients (r^2) were >0.99 for standard curves of all quantified compounds.

For each QC, the concentration was recalculated from the equation of the linear regression curve. The results of intra-day ($n=6$) and inter-day ($n=6$) validation are summarized in Table 1. The method is reproducible with coefficient of variation (C.V.) values between 2.2 and 9.7% for intra-day validation and 3.6 and 13.0% for inter-day validation for I3C, DIM and LTr_1, analyzed at a similar concentration range using UV detection, and C.V. values of between 4.2 and 13.5% for intra-day validation and 4.9 and 16.1% were obtained for the inter-day validation for ICZ, analyzed over a smaller concentration range, using fluorescence detection. Interestingly, when ICZ was validated in the absence of I.S. (i.e. using PAs and not PARs) the reproducibility was improved for this compound. Thus C.V. values achieved were between 1.7 and 5.3% (intra-day validation) and 1.9 and 9.7% (inter-day validation). ICZ was therefore further validated disregarding I.S. and using simple PA data. For all compounds, at all concentrations investigated, the chosen method yields standard accuracy values from 86.4 to 105.1% for intra-day validation and 92.0 to 112% for inter-day validation.

Using our established method, the recovery of I3C, DIM, LTr_1 and ICZ from plasma was found to be 89.5, 88.2, 50.0 and 84.9%, respectively.

The LOD of I3C, DIM, LTr_1 and ICZ was 30 ng/ml (204 n*M*), 30 ng/ml (122 n*M*), 100 ng/ml (266 n*M*) and 0.10 ng/ml (0.390 n*M*), respectively. The LOQ was determined to be 50 ng/ml for both I3C (340 n*M*) and DIM (203 n*M*), 150 ng/ml for LTr_1 (400 n*M*), and 0.15 ng/ml for ICZ (0.585 n*M*). The LOD and LOQ for I3C and DIM were slightly lower using fluorescence detection but linearity was much superior using UV detection and quantification over a wider concentration range was possible.

The method reported within the manuscript is currently being applied to comprehensive pharmacokinetic analyses involving oral and intravenous dosing of mice with I3C and DIM. The dose used in these studies (250 mg/kg) is calculated to be equivalent to a 1.35 g dose in an average (70 kg) adult human [24]. In a preliminary investigation, involving dosing of mice with I3C (250 mg/kg), chromatograms of plasma samples taken at 15 min and 60 min following dosing are depicted in Fig. 5. Peaks with retention times corresponding to I3C, DIM and LTr_1 standards had identical mass spectra to those compounds found in the acid reaction mixture (Fig. 2) and to those previously reported [10]. At the 15 min time-point, I3C and DIM can be detected at levels corresponding to 6210 ng/ml and 140 ng/ml, respectively (Fig. 5A), whereas at 60 min after dosing, I3C is below the limit of detection but DIM and LTr_1

Table 1
Results of intra- and inter-day validation in spiked mouse plasma

	LLOQ				LoQC				MeQC				HiQC			
	I3C	DIM	LTr_1	ICZ	I3C	DIM	LTr_1	ICZ	I3C	DIM	LTr_1	ICZ	I3C	DIM	LTr_1	ICZ
Selected conc. (ng/ml)	50	50	150	0.15	75	75	200	0.225	7500	7500	7500	22.5	12,500	12,500	12,500	37.5
Intra-day validation (*n*)	6				6				6				6			
Measured mean conc. (ng/ml)	44.2	44.2	147.2	0.15	64.8	67.4	198.6	0.21	7685	7301	7882	23.6	12,689	12,067	12,634	36.5
SD (ng/ml)	4.29	3.46	11.4	0.00	2.39	6.09	15.7	0.00	318.3	161.9	308.0	1.24	566.1	635.9	928.3	1.42
C.V. (%)	9.70	7.82	7.74	2.65	3.69	9.04	7.9	1.70	4.14	2.22	3.91	5.28	4.46	5.27	7.35	3.89
Accuracy (%)	88.5	88.4	98.1	97.5	86.4	89.9	99.3	91.3	102.5	97.35	105.1	104.7	101.5	96.5	101.1	97.2
Inter-day validation (*n*)	6				6				6				6			
Measured mean conc. (ng/ml)	48.9	47.3	166.9	0.17	82.9	81.4	184.0	0.25	7477	7510	7493	23.17	12,149	12,084	12,072	36.8
SD (ng/ml)	6.35	5.60	11.01	0.02	4.38	3.47	6.58	0.02	394.7	528.7	307.8	0.49	705.8	607.5	934.0	0.7
C.V. (%)	13.0	11.8	6.60	9.65	5.28	4.27	3.57	8.81	5.28	7.04	4.11	2.10	5.81	5.03	7.74	1.91
Accuracy (%)	97.9	94.7	111.3	112.4	110.5	108.5	92.0	111.3	99.7	100.1	99.9	103.0	97.2	96.7	96.6	98.1

ICZ analysis is validated without the use of internal standard. *n*, number of analytical runs; SD, standard deviation; C.V., coefficient of variation.

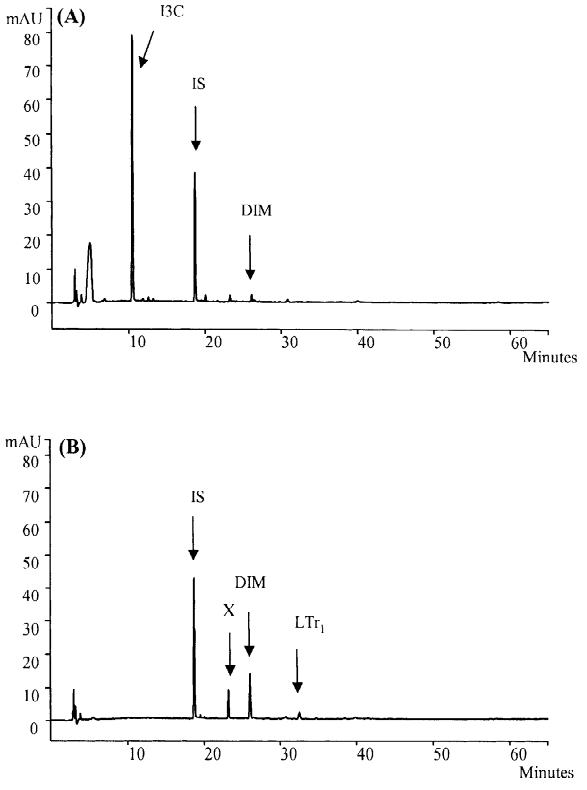

Fig. 5. HPLC chromatograms of mouse plasma samples taken (A) 15 min and (B) 1 h after a single oral administration of I3C (250 mg/kg). UV detection was at 280 nm. In (A), the concentration of I3C and DIM was 6210 ng/ml and 140 ng/ml, respectively. In (B), the concentration of DIM and LTr_1 was 958 ng/ml and 360 ng/ml, respectively. I3C, indole-3-carbinol; DIM, 3,3′-diindolylmethane; LTr_1, 1st linear trimer; IS, internal standard; X, unidentified metabolite.

are detected at levels corresponding to 958 and 360 ng/ml, respectively (Fig. 5B). No interfering peaks from plasma constituents have been observed in any samples and furthermore, there is good separation of a single (unidentified) metabolite (23 min, Fig. 5B) [$\alpha_{\text{I.S.-metabolite}} = 1.26$; $\alpha_{\text{metabolite-DIM}} = 1.15$]. ICZ was below the limit of quantification and no other acid condensation products were observed.

In conclusion, because of its extreme sensitivity to acid-catalyzed oligomerization, the bioanalytical determination of I3C is challenging and, although a number of HPLC methods have been described for the analysis of I3C and its condensation products [9,10,18,19,21], ours is the first fully validated method to be reported. It is the first method to demonstrate a clear separation of I3C and its acid condensation products of interest in a single chromatogram (Figs. 1 and 2) and the fully validated method, incorporating an internal standard and UV and fluorescence detection in tandem, offers a means to simultaneously and accurately quantify I3C and its oligomers of major interest in plasma. This, in turn, allows comprehensive pharmacokinetic and metabolic studies of I3C to be accomplished and future chemoprevention studies with I3C will require such a method to enable discrimination between indoles potentially responsible for the biological effects of I3C. The validation results presented in this paper, in combination with preliminary pharmacokinetic investigations, indicate that the assay as described fits all the necessary criteria: it is not only robust and reproducible but also accurate and practical.

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