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Determination of S-carboxymethyl-L-cysteine and some of its metabolites in urine and serum by high-performance liquid chromatography using fluorescent pre-column labelling

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ABSTRACT

Pre-column labelling techniques are described for the determination of S-carboxymethyl-L-cysteine (CMC) and its metabolites in urine and plasma samples by high-performance liquid chromatography (HPLC) without prior extraction. All substances containing an amino group were converted into fluorescent fluorenylmethyl derivatives with 9-fluorenylmethyloxycarbonyl chloride (FMOC). Deaminated or N-acetylated carbocysteine metabolites were coupled with 1-pyrenyldiazomethane (PDAM) to give fluorescent PDAM esters. Similar results were obtained with the two commercially available and stable diazomethane derivatives PDAM and 9-anthryldiazomethane (ADAM). Following double derivatization with PDAM and FMOC, in a single chromatographic run with two fluorescence detectors connected in series, amines and amino(carboxylic) acids could be detected by their FMOC residues and, simultaneously, carboxylic acids were detected as fluorescent PDAM esters. The (*R*) and (*S*) enantiomers of the sulphoxides of CMC, of methylcysteine and of N-acetyl CMC were separated, although the reversed-phase HPLC system did not contain a chiral additive or stationary phase designed for the separation of enantiomers. The methods do not include liquid extraction steps and can therefore be performed either manually or automatically using an HPLC autosampler. These methods were used for the investigation of a disputed pharmacogenetic polymorphism of S-oxidation of CMC in humans, which until now has most often been studied using paper chromatography. The described techniques were applied to the determination of CMC and its metabolites in human urine and plasma samples.

INTRODUCTION

S-Carboxymethyl-L-cysteine (carbocysteine; CMC) is a mucolytic drug used in the treatment of chronic bronchitis. The metabolism of CMC is known to be very complex [1]. It may be oxidized to (*R*,*S*)-S-carboxymethylcysteine sulphoxide, decarboxylated, or N-acetylated. S-Oxidation has also been described for the acetylated or decarboxylated metabolites. It has been reported [2] that humans exhibit considerable inter-individual differences in the sulphoxidation of this drug and its metabolites. Recent reports suggest that differences in S-oxidation of CMC might be correlated with the incidence of severe diseases such as primary biliary cirrhosis [3], Alzheimer's disease [4] or the occurrence of severe side-effects

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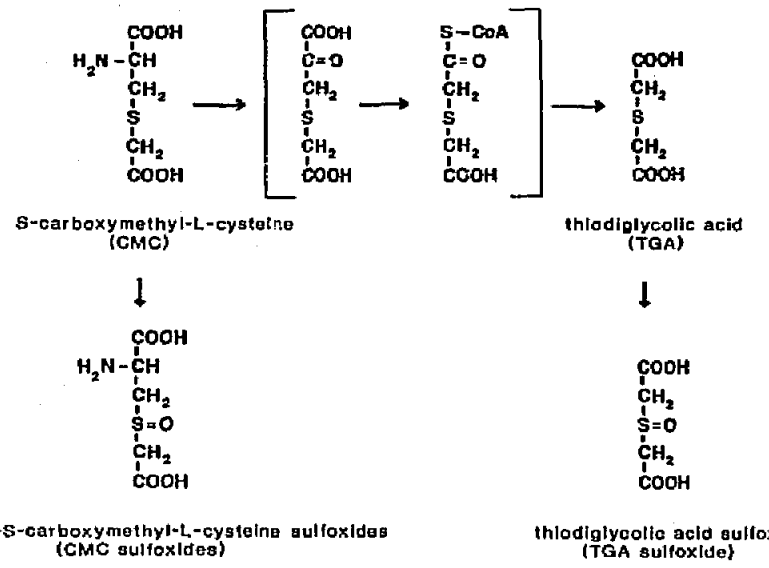

Fig. 1. Structures of some of the compounds determined by the described methods and their metabolic relationships. The intermediates (shown in brackets) are hypothetical and require further experimental confirmation. All sulphoxides with two different residues exist as enantiomers.

during therapy with the antirheumatic D-penicillamine [5]. As the ratios between different CMC metabolites observed in these studies and obtained using paper chromatography could not be confirmed by others [6,7], further investigation with a highly sensitive and specific analytical alternative is necessary. The metabolic pathways of the compounds studied are given in Fig. 1.

Methods for the determination of CMC, but not of its metabolites, by reversed-phase ion-pair chromatography with column switching following pre-column derivatization with *o*-phthalaldehyde have been described [8,9]. The serum kinetics of CMC have been analysed by gas chromatography (GC) [10]. This method has also been used to identify thiodiglycolic acid (TGA) as a metabolite of CMC in urine [11]. GC is, however, not optimal for the quantification of some unstable metabolites, such as the S-oxides. A ^{13}C-nuclear magnetic resonance (NMR) method has been developed, which may allow the determination of the ^{13}C-labelled drug in urine samples without prior sample preparation steps [12].

The goal of the work reported here was to develop a high-performance liquid chromatographic (HPLC) method that would allow a study of inter-individual CMC metabolism in humans. For several reasons CMC and its metabolites are difficult to determine. Firstly, it is difficult to detect aliphatic amino acids and aliphatic carbonic acids directly in urine due to the presence of endogenous compounds that strongly absorb low-wavelength UV light. Secondly, as a result of their hydrophilicity, solvent extraction of these metabolites from urine is impossible. Two methods were developed for the determination of CMC and its metabolites without prior extraction from urine.

One method is based on derivatization with 9-fluorenylmethylchloroformate (FMOC), introduced into amino acid analysis by Einarsson *et al.* [13]. It allows the determination of metabolites containing an amino group in urine and plasma samples. It was possible to separate the optical enantiomers of the S-oxides using the FMOC method. N-Acetylated and deaminated metabolites were determined using the reagent 1-pyrenyldiazomethane (PDAM), previously used in the determination of fatty acids.

Both methods produced highly fluorescent and stable products, which could be separated by gradient reversed-phase HPLC. During the optimization of the FMOC and PDAM methods it became apparent that both derivatization reactions can be performed in a single procedure, allowing the detection of amines, amino acids and carboxylic acids in a single chromatographic run. Methods of FMOC and PDAM single and double derivatizations are therefore described and applied to the determination of metabolites (or presumed metabolites) of CMC in urine and serum samples.

EXPERIMENTAL

Apparatus

The chromatography was performed with a Series 4 gradient liquid chromatograph (from Perkin-Elmer, Norwalk, CT, USA), equipped with an ISS-100 autosampler (Perkin-Elmer). Fluorescence was detected using an RF-530 and an RF-535 spectrofluoromonitor and two CR3A integrators (Shimadzu, Kyoto, Japan). Samples derivatized with FMOC only were separated on a Hypersil ODS column (Shandon, UK; 5 μm particles, 250 mm × 4.6 mm I.D.). The PDAM and double-derivatized samples were separated on an Ultracarb ODS column (Phenomenex, USA; 5 μm particle size, 150 mm × 4.6 mm I.D.).

Chemicals

FMOC, methylcysteine, methionine, methionine sulphoxide and methionine sulphone were obtained from Sigma (St. Louis, MO, USA). Carboxyethylcysteine, carboxymethylcysteine and 1-octanesulphonic acid were obtained from Fluka (Buchs, Switzerland). Thiodiglycolic acid (TGA) was purchased from Aldrich (Steinheim, Germany). PDAM and 9-anthryldiazomethane (ADAM) were obtained from Molecular Probes (Eugene, OR, USA) and were used without further purification. N-Acetylalanine was purchased from Serva (Heidelberg, Germany). Triethanolamine was obtained from Pierce (Steinheim, Germany). (*R,S*)-Carboxymethylcysteine sulphoxide and sulphone, N-acetylcarboxymethylcysteine, N-acetylcarboxymethylcysteine sulphoxide, as well as the sulphoxides and the sulphone of TGA were synthesized as described later. Acetonitrile from Merck (Darmstadt, Germany) was of HPLC grade. All other chemicals, obtained from the same company, were of analytical-reagent grade.

FMOC method

Reagent solutions and eluents. The derivatization buffer contained 3.09 g of boric acid in 100 ml of HPLC-grade water adjusted to pH 7.7 with 30% sodium hydroxide. The derivatization reagent was prepared by dissolving 129 mg of FMOC in 50 ml of acetone to give a concentration of 10 m*M*. The ammonium acetate buffer for chromatography was prepared by the addition of 2 ml of 25% ammonia solution to 1 l of doubly distilled water, the pH was adjusted to 3.8 and dimethylformamide was added to give a final concentration of 4%.

Derivatization. A 5-μg sample of carboxyethylcysteine (50 μl of a 100 μg/ml aqueous solution) was added as an internal standard to 50-μl samples. The standards were added to 50-μl portions of blank urine or plasma samples for calibration. These samples were dried in a Speed Vac (Savant Instruments, Farmingdale, NY, USA) vacuum centrifuge and redissolved by the addition of 400 μl of borate buffer and 400 μl of FMOC solution. The resulting mixture was allowed to react for 50 s. After stopping the derivatization by the addition of 35 μl of 0.5 *M* ammonium acetate solution, the samples were ready for injection.

Separation and quantitation. The separation was carried out by gradient elution on the Hypersil ODS column described under *Apparatus*. The eluent was increased linearly from a buffer/acetonitrile ratio of 90:10 (v/v) to a ratio of 65:35 (v/v) over 65 min. The flow-rate was 0.8 ml/min and the oven temperature 40°C. The wavelengths for excitation and emission were 260 and 305 nm, respectively.

PDAM method

Reagent solutions and eluents. The buffer for derivatization contained 100 m*M* phosphoric acid adjusted to pH 4.0 with sodium hydroxide in 50% propan-2-ol. The PDAM reagent solution was prepared freshly before use by dissolving 3.5 mg/ml of the commercially available reagent (without further purification) in ethyl acetate to give a concentration of 15 m*M*. The eluent consisted of a 25 m*M* triethylamine (TEA)–phosphate buffer, pH 2.4, containing 5% tetrahydrofuran and 20% methanol.

Derivatization. A 2.5-μg sample of N-acetylalanine (internal standard) was added to 50-μl samples of urine or plasma which were subsequently evaporarated to dryness under vacuum and then redissolved in 300 μl of phosphate buffer. A 300-μl volume of 1-octanesulphonic acid solution (20 μ*M* in propan-2-ol) and 300 μl of reagent solution were then added. The mixture was gently shaken at 40°C for 30 min and diluted to 1.5 ml with propan-2-ol.

Separation and quantification. The separation was performed with gradient elution on the Ultracarb ODS column (see *Apparatus*). The gradient increased linearly from a ratio of 80:20 (v/v) TEA buffer/acetonitrile to 65:35 (v/v) over 70 min at a flow-rate of 0.8 ml/min and a column temperature of 50°C. The wavelengths for excitation and emission were 340 and 380 nm, respectively. The metabolites were quantified by peak-height measurements. The reaction schemes of the studied compounds are given in Fig. 2.

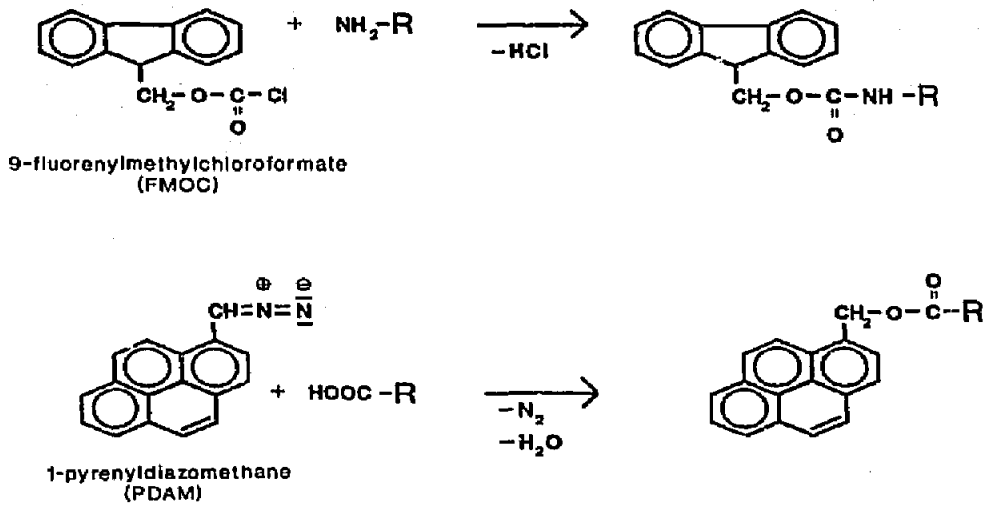

Fig. 2. Structures of the reagents FMOC and PDAM and their reaction with amines and carboxylic acids.

PDAM–FMOC double derivatization

The reagent solutions were the same as described for the single derivatizations. For the chromatographic separation, a 40 m*M* TEA–phosphate buffer adjusted to pH 3.2 with phosphoric acid containing 5% tetrahydrofuran and 10% methanol was used.

Derivatization. A 1.25-μg sample of N-acetylalanine (internal standard) was added to 25 μl of urine or plasma. Standard concentrations of the respective calibration solutions were added to blank plasma and urine samples, the mixtures were evaporated to dryness in a Speed-Vac concentrator and then, after redissolving in 150 μl of phosphate buffer, 150 μl of 1-octanesulphonic acid solution and 150 μl of PDAM reagent solution were added. After 30 min at 40°C, 150 μl of propan-2-ol were added and the mixture was adjusted to pH 8.0 by the addition of 400 μl of 0.5 *M* borate buffer. The FMOC derivatization was carried out by the addition of 400 μl of FMOC reagent as described previously. A 10-μl aliquot of the final derivative solution was injected into the HPLC system.

Separation and quantitation. Chromatographic separation was carried out by gradient elution on the Ultracarb ODS column. Elution was performed with a linear gradient from a ratio of 75:25 (v/v) TEA buffer/acetonitrile to 50:50 (v/v) over 80 min at a flow-rate of 0.8 ml/min and a column temperature of 60°C.

Synthesis of reference compounds

(R)- and (S)-S-carboxymethylcysteine sulphoxides. To a solution of 10 g (56 mmol) of CMC in 250 ml of formic acid were added 6.3 ml (56 mmol) of a 30% aqueous hydrogen peroxide solution. The mixture was stirred overnight at 4°C. This solution was diluted to 500 ml with doubly distilled water and subsequently evaporated. Yield 95%; m.p. 173°C, published value [14] 173–175°C; $C_5H_9NO_5S$ (M_r 195.2) calculated C 30.8, H 4.65, N 7.2, found C 30.7, H 4.66, N 7.2; ^1H

NMR (300 MHz, D_2O/NaOD) ABM system, $\delta_A = 3.08$, $\delta_B = 3.27$, $\delta_M = 3.69$ (3H, $J_{A/B} = 13.3$ Hz; $J_{A/M} = 7.0$ Hz; $J_{B/M} = 6.6$ Hz).

Fractional crystallization to separate the *R,S* enantiomers of S-carboxymethylcysteine sulphoxide was carried out according to Meese [14]. The optical purity of the two isomers was greater than 99%.

TGA sulphoxide. To a solution of 10 g (67 mmol) of TGA in water were added 8.4 ml (74 mmol) of 30% aqueous hydrogen peroxide solution. The mixture was stirred overnight at 4°C followed by evaporation after dilution with water to 100 ml. Yield 95%; m.p. 120°C (hygroscopic); $C_4H_6O_5S$ (M_r 166.15) calculated C 28.9, H 3.61, found C 28.3, H 3.71. ^1H NMR (300 MHz, D_2O) AB system, $\delta_A = 4.01$, $\delta_B = 4.15$, (4H, $J_{A/B} = 15.3$ Hz).

Sulphones of CMC, methylcysteine and TGA. A solution of 10 mmol of the corresponding sulphide in 1.5 ml (15 mmol) of hydrogen peroxide and 6 ml (130 mmol) of formic acid was allowed to react at 60°C for 2 h followed by evaporation after dilution with water to 100 ml.

CMC sulphone. Yield 90%; m.p. 180°C; ^1H NMR (300 MHz, D_2O), $\delta = 3.65$ (H 1, m_z), $\delta = 3.98$ (H-2, m_z), $\delta = 4.87$ (H-3, m_z).

TGA sulphone. Yield 90%; m.p. 155°C; ^1H NMR (300 MHz, D_2O), $\delta = 4.52$ (4H, s).

Methylcysteine sulphone. Yield 95%; m.p. 178°C; ^1H NMR (300 MHz, D_2O, NaOD) ABM system, $\delta_A = 3.42$, $\delta_B = 3.67$, $\delta_M = 3.83$ (3H, $J_{A/B} = 14.4$ Hz; $J_{A/M} = 8.5$ Hz; $J_{B/M} = 3.6$ Hz).

N-Acetyl-CMC. A solution of 10 g (56 mmol) of CMC in 6 ml of acetic anhydride was allowed to react at room temperature for 12 h. After dilution with water to 50 ml, the reaction mixture was percolated through a column (5 cm × 2.5 cm I.D.) of Dowex 50 WX 8, mesh size 100–200 (Serva) to retain free amino groups. The collected fraction was evaporated under vacuum, leaving a syrupy residue which solidified on dissolution in methanol and crystallization with acetone. Yield 62%; $C_7H_{11}NO_5S$ (M_r 221.2) calculated C 38.00, H 5.012, N 6.33, found C 38.1, H 5.019, N 6.32; ^1H NMR (300 MHz, D_2O, NaOD) ABM system, $\delta_A = 3.91$, $\delta_B = 3.02$, $\delta_M = 4.35$ (3H, $J_{A/B} = 13.7$ Hz; $J_{A/M} = 8.3$ Hz; $J_{B/M} = 4.3$ Hz), $\delta = 3.27$ (2H, s) $\delta = 2.06$ (3H, s). The acetylated product could be completely converted to CMC by acid hydrolysis (6 *M* hydrochloric acid, 100°C, 1 h) as determined by the chromatographic procedures described here.

N-Acetyl-(R,S)-CMC sulphoxide. Starting with racemic or pure (*R*) and (*S*) enantiomers, the acetylation was carried out as described above. Yield (after final purification) 40%; m.p. 153°C; $C_7H_{11}NO_6S$ (M_r 237.2) calculated C 35.41, H 4.637, N 5.90, found C 35.40, H 4.640, N 5.91; ^1H NMR (300 MHz, D_2O, NaOD) ABM system, $\delta_A = 3.21$, $\delta_B = 3.48$, $\delta_M = 4.62$ (3H, $J_{A/B} = 13.7$ Hz; $J_{A/M} = 8.3$ Hz; $J_{B/M} = 5.3$ Hz), $\delta = 2.01$ (3H, s).

RESULTS AND DISCUSSION

Complete separation of the (*R*)- and (*S*)-CMC sulphoxides (as FMOC derivatives) was obtained with different C_{18} reversed-phase materials tested with buffers at pH values between 3.8 and 4.5. The separation of the enantiomers of S-methylcysteine was complete only at pH values between 5 and 6. Also, the separation of S-methylcysteine and its sulphoxides from other endogeneous compounds was superior with 50 m*M* potassium phosphate buffer, pH 6, containing 4% tetrahydrofuran at 50°C.

As illustrated in Fig. 3, baseline separation of the S-oxides of CMC has been achieved. Similarly, the sulphoxides of S-methylcysteine were completely separated when the acetate buffer was adjusted to pH 5.5. Quantification of (*R*)- and (*S*)-CMC sulphoxides by the described technique was sensitive enough to allow the analysis of the human serum kinetics of these two minor metabolites of CMC in humans. The maximum serum concentrations of these metabolites were about 35 ng/ml (±10 ng/ml S.D.) for the (*S*)-enantiomer and 90 ng/ml (±30 ng/ml S.D.) for the (*R*) enantiomer following an oral dose of 1.125 g of CMC as investigated in ten healthy humans.

The analysis of S-oxidation of sulphide-containing drugs, as well as endogenous amino acids (and CMC obtained after the carboxymethylation of cysteine in amino acid analysis of proteins and peptides), may also be of interest in other areas of research. For example, the formation of methionine sulphoxides has been described as a consequence of the incorrect handling of biologically active peptides. (*R*)- and (*S*)-sulphoxides of methionine, however, could not be separated with the reversed-phase columns described here.

Amino acid derivatives such as the compounds described here can only be determined at lower concentrations by the use of derivatization methods. Higher concentrations can nevertheless be determined using reversed-phase or ion-exchange HPLC combined with short-wavelength UV-absorbance (200 nm) detection (chromatograms not shown). Different pre-column derivatization methods and reagents were investigated by this group for the derivatization of urine and plasma samples. The initial results were obtained with the chromogenic reagent 4-dimethylaminoazobenzene sulphonylchloride (DABS) [6] and the homologous fluorescent reagent 5-dimethylamino-1-naphthalenesulphonyl chloride. However, the FMOC derivatization procedure is preferred now over these reagents because of its simplicity. The 0.5 *M* borate buffer used in the FMOC method makes the reaction insensitive to pH differences in the urine samples, and therefore no prior pH adjustment of the urine samples is required.

The omission of any extraction steps prior to or following the derivatization procedures allowed automated derivatization and aberrations due to substance-specific extraction yields to be circumvented.

The new class of relatively stable diazomethane derivative reagents [15] are also easy to use and may find even more analytical applications in the future. For

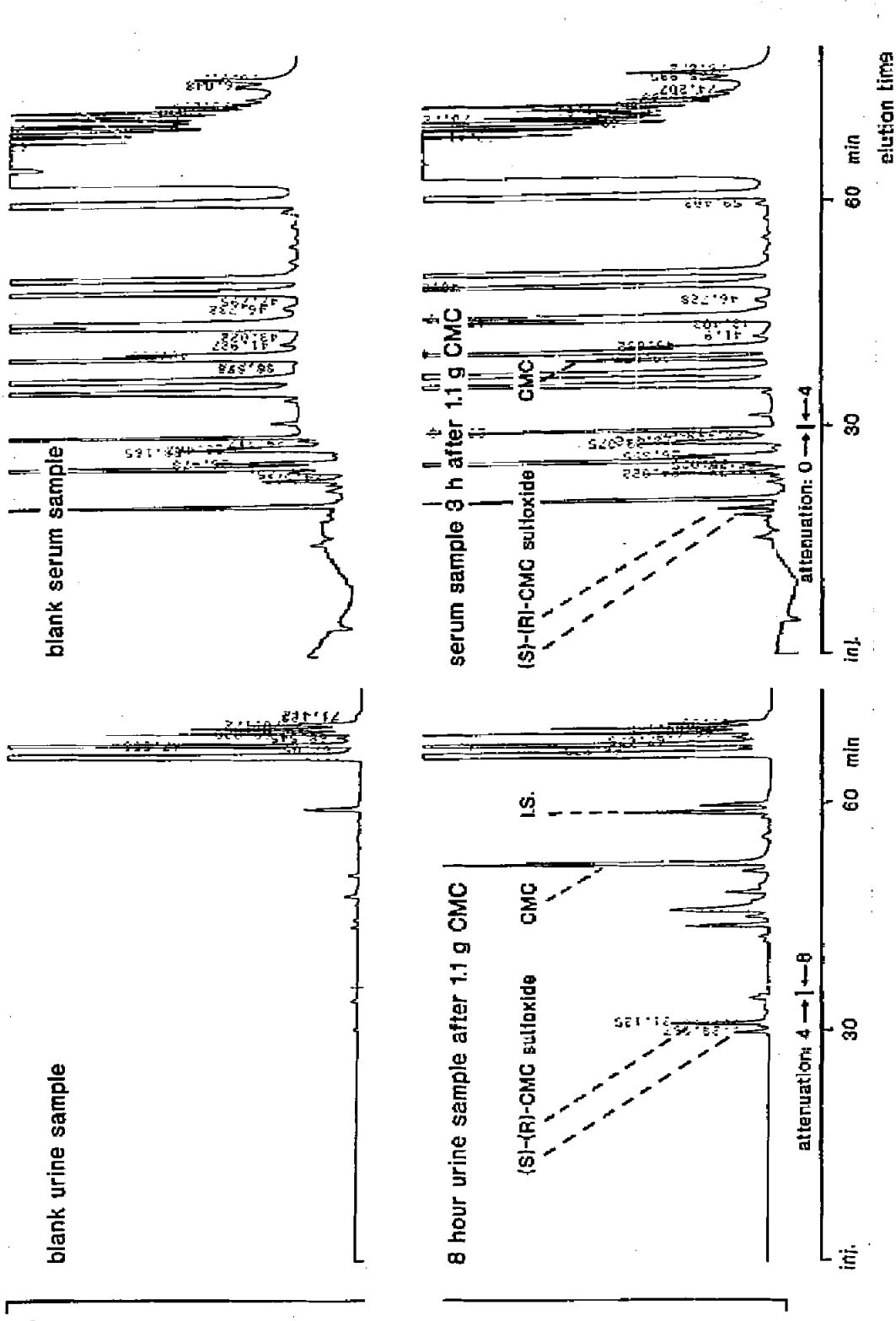

Fig. 3. Typical chromatograms of urine and serum samples before and after the ingestion of 1.1 g of CMC. Note that the amount of CMC sulphoxides is considerably smaller than that of CMC and the recorder attenuation therefore had to be changed by factors of 8 and 16 for the urine and serum samples, respectively. A linear gradient from 10 to 35% acetonitrile over a period of 65 min was used for the elution of the urine samples, whereas a more shallow gradient from 15 to 30% acetonitrile over 65 min was used for the serum samples to ensure the separation of CMC from other acidic amino acids.

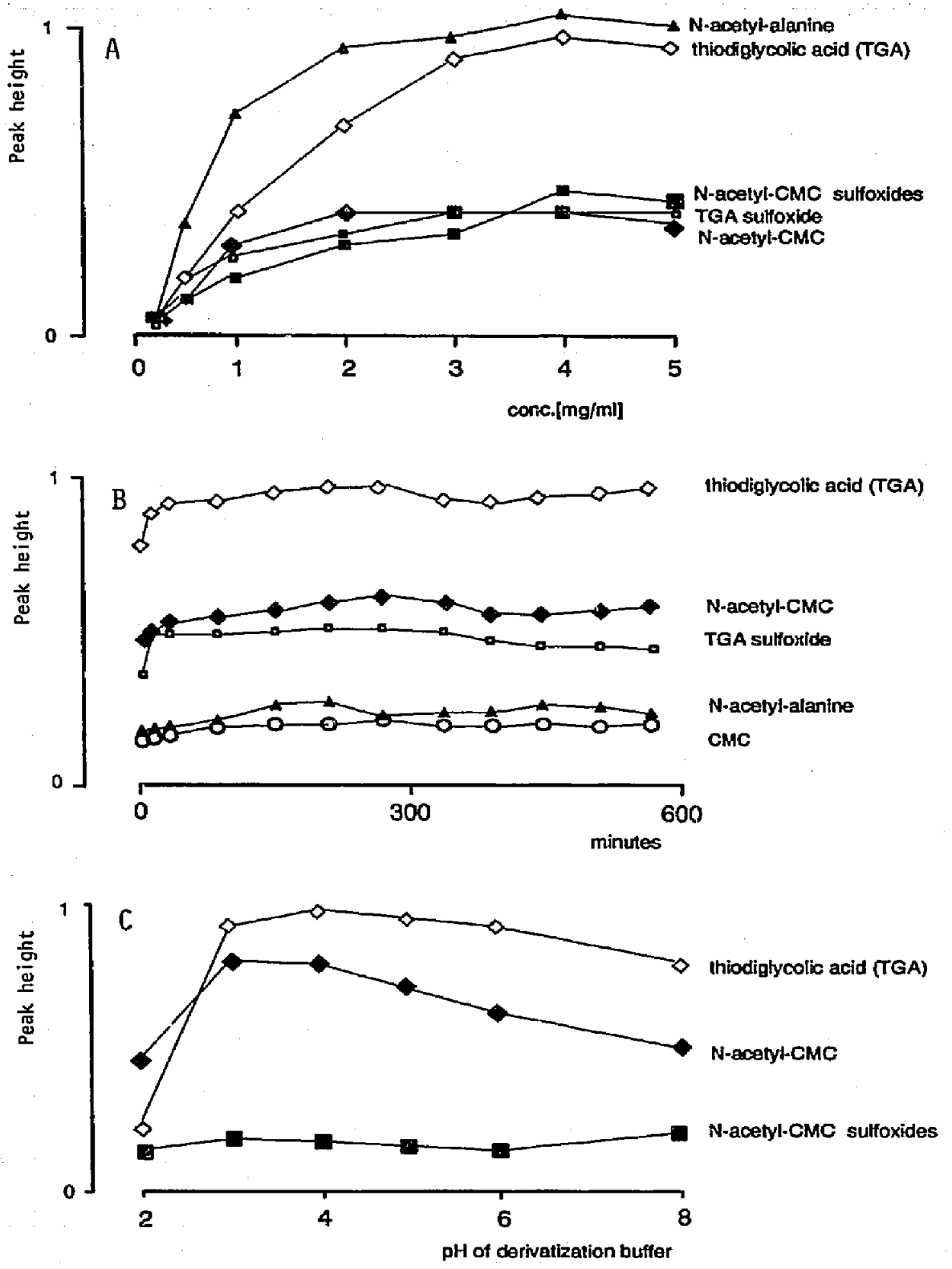

Fig. 4. Evaluation of derivatization conditions. (A) Concentration of PDAM required when using 50-μl urine samples spiked with 200 μg/ml of the respective reference compounds. The derivatization conditions were otherwise as described under Experimental. (B) Derivatization with PDAM as a function of reaction time. Even at room temperature, more than 70% of the final peak heights were obtained after 5 min with all compounds tested. The derivatives were stable at room temperature over a period of 48 h as tested by repetitive injection. (C) pH dependence of derivatization with PDAM.

TABLE I

RESULTS OF THE INTRA- AND INTER-ASSAY VARIANCE AS OBTAINED WITH FROZEN SAMPLES OF A SPIKED BLANK URINE

Percentage values in brackets indicate the deviations from the expected concentrations. Note that the evaluated quantitation ranges do not represent the lower limit of sensitivity, but merely the range of interest in these studies.

Compound	Concentration expected (μg/ml)	Intra-assay			Inter-assay			Quantification range (μg/ml)
		Mean found (μg/ml)	C.V. (%)	n	Mean found (μg/ml)	C.V. (%)	n	
FMOC pre-column derivatization method								
CMC	184.8	190.0 (+2.8%)	2.42	12	181.7 (−1.7%)	5.68	8	0.1–1000
S-(−)-CMC sulphoxide	4.32	4.08 (−5.6%)	4.12	10	4.49 (+3.9%)	9.71	8	0.1–1000
R-(+)-CMC sulphoxide	6.16	6.39 (+3.7%)	6.39	10	6.36 (+3.2%)	8.85	8	0.1–1000
PDAM pre-column derivatization method								
AcCMC	54.0	48.0 (−11.1%)	6.3	8	59.8 (+10.7%)	32.8	12	5–1000
AcCMC sulphoxide	56.5	48.4 (−14.3%)	7.2	6	47.7 (−15.6%)	27.8	10	5–1000
TGA	47.0	47.5 (+ 1.1%)	2.3	6	41.6 (−12.4%)	18.5	12	5–1000
TGA sulphoxide	78.5	85.4 (+ 8.8%)	6.9	6	91.5 (−16.6%)	11.9	10	5–1000

this purpose of the direct derivatization of biological fluids, the derivatization yields were evaluated as a function of reagent concentration, reaction time and temperature and H^+ concentration, as well as urine–matrix influences (Fig. 4).

Plateau reaction yields were seen at reagent concentrations (Fig. 4A) of 4 mg/ml using 50 μl of urine or plasma and 300 μl of the 15 m*M* reagent solution. The maximum solubility of the reagent was 20 m*M* at 40°C. These reaction yields were evaluated in blank urine samples from different persons with standard compounds added. As in all the procedures described here, small urine or serum volumes (50 μl or below) were derivatized with a large excess of reagent, as the derivatization reagent reacts not only with the substances of interest, but also with many endogenous compounds.

Although at room temperature the maximum derivatization yield needed a reaction time of 2 h, more than 70% of the final peak heights were achieved after an incubation period of 5 min at room temperature (Fig. 4B). Irrespective of the reaction time, the yields of the different compounds increased proportionally to one another. This allowed derivatization times even shorter than 30 min, especially as internal standards can always be included. The derivatives were stable for more than 24 h in the buffer used for derivatization. The reaction yields (Fig. 3) were optimal at pH values of around 4, similar to those described previously [15] for the determination of fatty acids.

Concentrations of the calibration solutions for the PDAM method were between 5 and 1000 μg/ml, as the relevant metabolite concentrations (>0.5% of the doses) after the administration of a 1.1-g dose of CMC are expected to be above 5 μg/ml. The reproducibility of these methods was tested by analysing urine samples spiked with standard substances in the concentration range expected in human urine samples. The results are summarized in Table I.

Following sequential derivatization of the aminocarboxylic acids with PDAM and with FMOC, only FMOC derivatives were found (Fig. 5). The reaction yields of the (non-amino) carboxylic acids, *e.g.* TGA, were identical whether derivatized only with PDAM or sequentially derivatized with both reagents. Steric interactions were ruled out as the reason for the hindrance of double derivatization by mono- and double-derivatizing aminohexanecarbonic acid, a molecule in which the reactant groups are far apart. Similar to the other amino acids, this compound yielded the respective PDAM esters in the mono derivatization reaction, but only the FMOC derivatives in the double derivatization reaction. The PDAM ester bond obviously becomes unstable when amino and carboxylic group containing compounds are derivatized in the second step with FMOC at pH 8 (Fig. 6).

The two homologous reagents PDAM and ADAM, gave essentially the same results (Fig. 7). Similar to PDAM, double derivatization only resulted in FMOC derivatives for the aminocarboxylic acids. Using these methods, 10–40% of a given oral CMC dose was found as the original substance, 5–30% was found as TGA, and 3–12% as TGA sulphoxide, in an 8-h pooled urine sample. The CMC

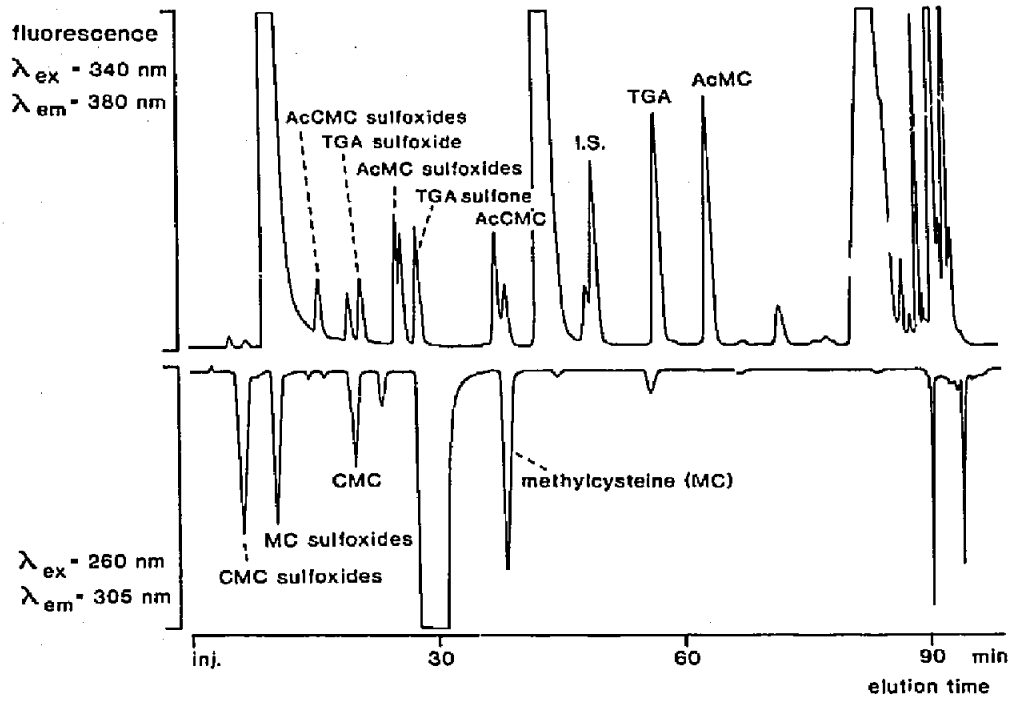

Fig. 5. Separation and detection of the reference compounds in a single chromatographic run using two fluorimeters connected in series. In the upper chromatogram all compounds containing a carboxylic group were detected as PDAM esters. In the lower chromatogram, the amines and amino acids were detected as FMOC derivatives. Almost no peaks had identical retention times. During the FMOC reaction step in the double-derivatization procedure the PDAM group is removed from the amino acids. Abbreviations: CMC = S-carboxymethyl-L-cysteine; MC = S-methyl-L-cysteine; AcCMC = N-acetyl-S-carboxymethyl-L-cysteine; TGA = thiodiglycolic acid; I.S. = internal standard = N-acetylalanine; AcMC = N-acetyl-S-methylcysteine.

sulphoxides, previously assumed to be a good indicator of the S-oxidation capacity, could always be detected, but only in amounts which were quantitatively ranging around 1% of the dose in any of thirty volunteers.

The specific quantitation of the complex pattern of CMC and more than eight of its metabolites in urine samples (as described previously using paper chromatography [1,2]) is not easy because of the similarity of these compounds with many endogenous substances. Using carboxyl group derivatization, the peak shape for all amino acids is not as good as that usually obtained following amino group derivatization (see, for example, Fig. 3). If separation could be improved further, amino acid analysis may be replaced by carboxylic group derivatization, which has advantages over current methodology only in the rare cases of amino group blocked amino acids.

Beyond the special questions of CMC and its metabolism, these authors believe that the amino and carboxylic group double-derivatization procedure described here may be useful for the determination of a variety of endogenous

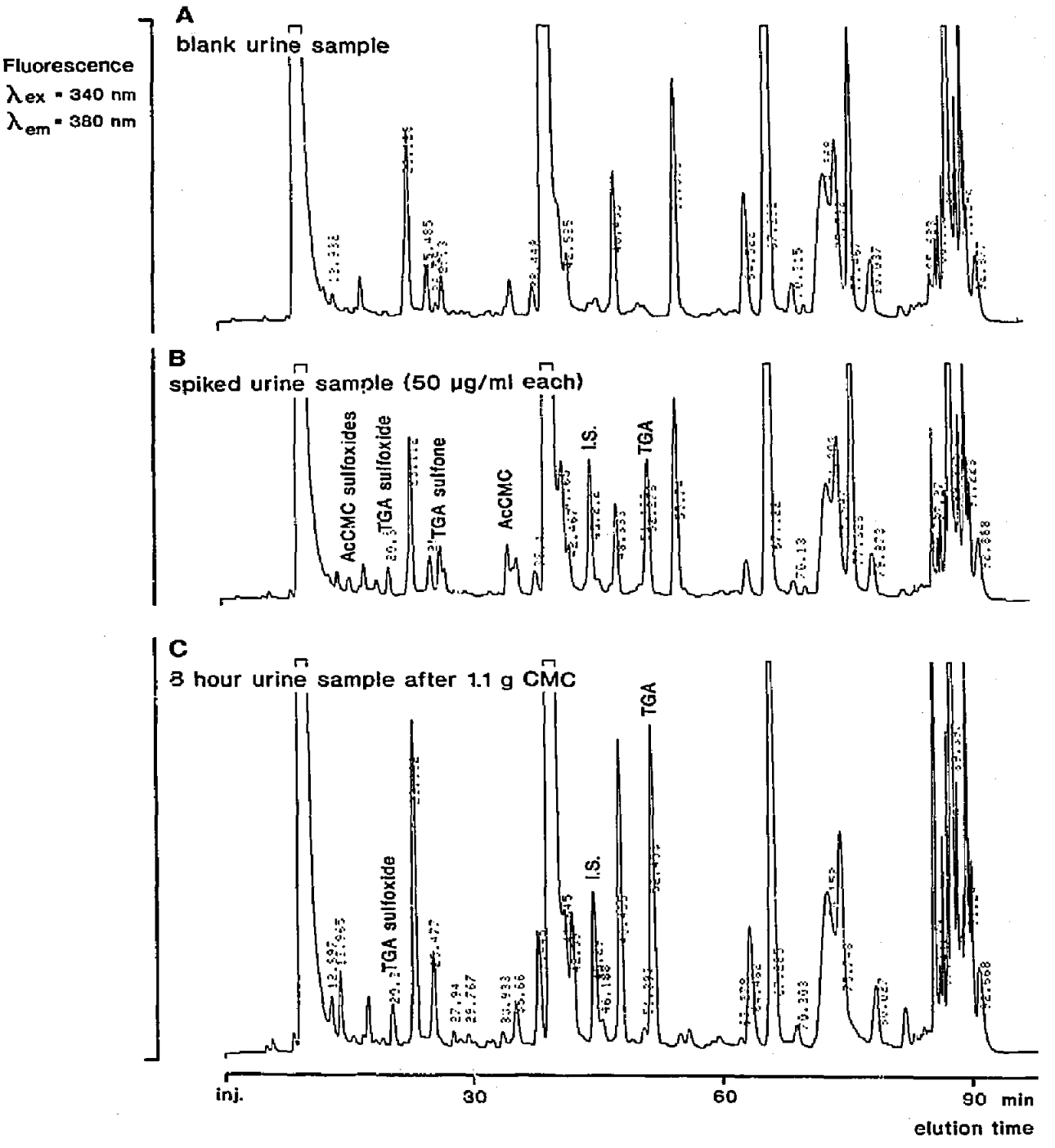

Fig. 6. Chromatograms of urine samples after derivatization with PDAM–FMOC and detection of PDAM esters (excitation 340 nm, emission, 380 nm). (A) Blank urine sample. (B) Blank urine sample spiked with 50 µg/ml of each of the following standards: AcCMC sulphoxides, TGA, TGA sulphoxide, TGA sulphone, AcCMC and N-acetylalanine as I.S. The elution gradient is described under Experimental (identical to that in Fig. 5). (C) Urine sample collected for 8 h after oral administration of three capsules of CMC (1.125 g) to a healthy volunteer. Note that the relatively large peaks of TGA and TGA sulphoxide can be clearly identified. Small peaks with the retention time of TGA were also seen in some blank urine samples.

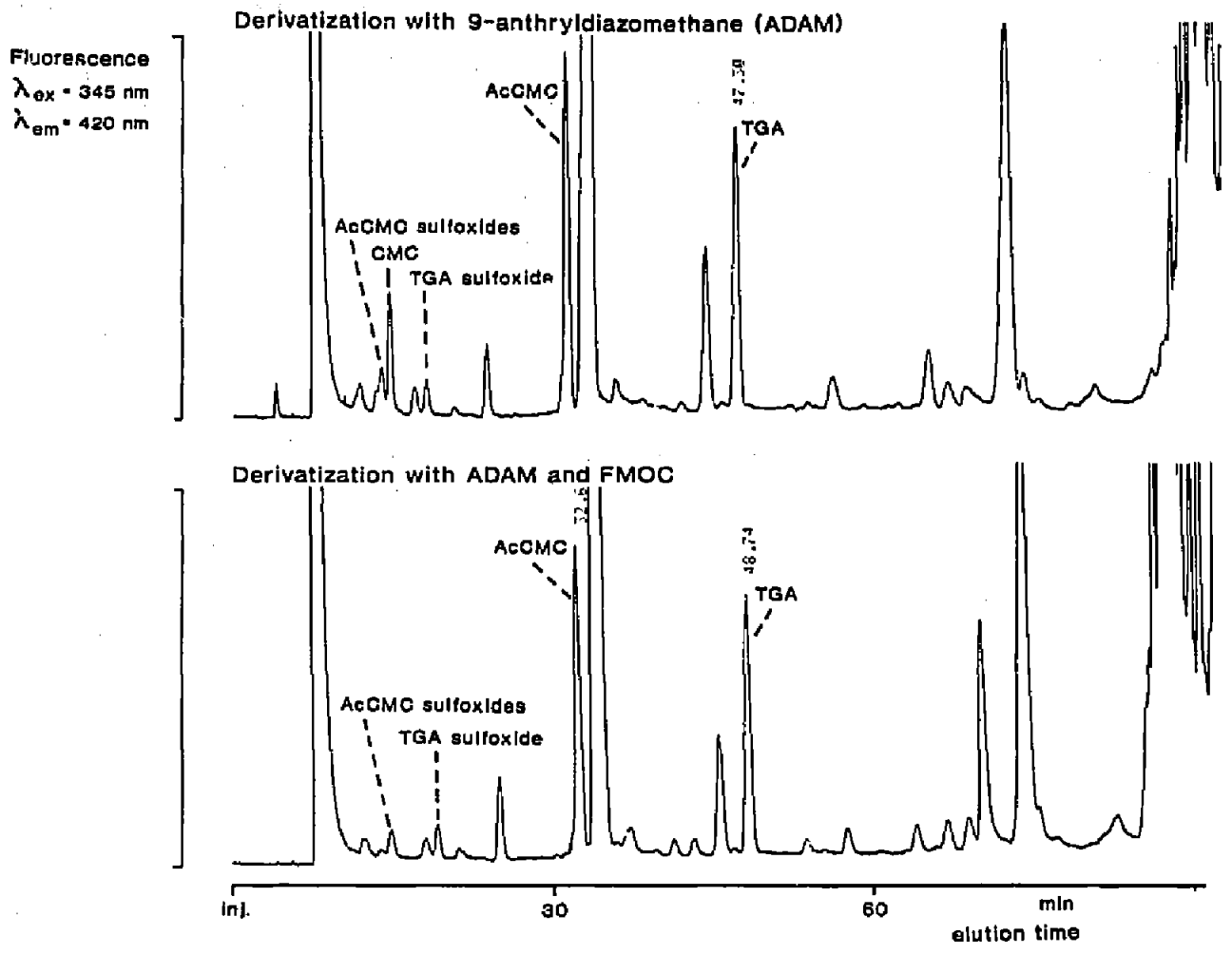

Fig. 7. Chromatograms of a urine sample spiked with 50 μg/ml of each of the following standards: AcCMC sulphoxides, CMC, TGA sulphoxide, AcCMC and TGA. The upper chromatogram was obtained by single derivatization with ADAM and the lower chromatogram was obtained after ADAM–FMOC double derivatization.

compounds as well as for the quantitation of some other drugs in biological samples.

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