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Note

Fluorescence detection of cystine by *o*-phthalaldehyde derivatisation and its separation using high-performance liquid chromatography

J.D.H. COOPER* and D.C. TURNELL

Biochemistry Department, Coventry and Warwickshire Hospital, Stoney Stanton Road, Coventry (Great Britain)

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The *o*-phthalaldehyde (OPA)—2-mercaptoethanol (MCE) derivatisation of primary amines [1] is ideally suited to the detection of amino acids in physiological fluids using liquid chromatography. This derivatisation step may be successfully performed using either a post-column [2] or pre-column [3] technique. The production of fluorescent derivatives when reacting OPA—MCE with primary amines is by the formation of 1-alkylthio-2-alkylisoindoles [4]. Since the reaction only proceeds in the presence of MCE, cystine is reduced to its monomer, cysteine. One disadvantage of the OPA—MCE reaction is that cysteine, unlike other amino acids containing primary amines, yields a derivative with minimal fluorescence [1]. This problem may be overcome by oxidising cystine or cysteine to cysteic acid, which when reacted with OPA—MCE yields a derivative with fluorescent properties comparable to those of other amino acid derivatives [1]. Because the conditions for these two reactions are vastly different, one being oxidising and the other reducing, it is difficult to obtain between-sample reproducibility of amino acid derivatisation with OPA—MCE following oxidation of cystine and cysteine.

This paper reports a simple procedure to overcome this problem by pretreating cysteine with iodoacetic acid before OPA—MCE derivatisation. This produces a cysteine derivative with a fluorescence intensity similar in magnitude to other amino acid OPA—MCE derivatives. This technique permits rapid chromatographic separation of cysteine OPA—MCE derivative using high-performance liquid chromatography (HPLC) and its quantification in biological samples.

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EXPERIMENTAL

Apparatus

The HPLC system used comprised an Altex pump and a 150 mm × 4.6 mm I.D. column pre-packed with 5-μm diameter Ultrasphere ODS (Anachem, Luton, Great Britain) and a Schoeffel FS970 fluorescence detector (Kratos, Manchester, Great Britain) using an excitation wavelength of 230 nm and an emission cut-off filter of 417 nm. The analytical column was fitted with a pre-column 70 mm × 2 mm I.D., packed with 25—37 μm diameter CO:PELL ODS (Whatman, Maidstone, Great Britain). Injections were made using a Rheodyne valve fitted with a 20-μl loop. Quantification of analytes was performed using an SP4100 integrator (Spectra-Physics, St. Albans, Great Britain).

Reagents

Unless otherwise stated all chemicals were analytical grade obtained from BDH (Poole, Great Britain). All solvents were filtered prior to use through a Millipore 0.5-μm filter (Millipore, London, Great Britain). Iodoacetic acid and MCE were obtained from Aldrich (Gillingham, Great Britain) and the OPA was Sepramar grade from BDH. The OPA—MCE was prepared in accordance with Lindroth and Mopper [3]. All amino acids were obtained from Sigma London (Poole, Great Britain).

Five standard solutions were prepared containing 50, 100, 150, 200 and 250 μ*M* cystine by diluting in water, a 10 m*M* solution of cystine in 0.1 *M* hydrochloric acid.

Chromatographic conditions

Optimum chromatographic conditions were established as follows: isocratic solvent, 50 m*M* disodium hydrogen phosphate buffer (pH 7.4 ± 0.1)—acetonitrile (89:11).

Derivatisation

The following volumes of reagents were used to optimise the derivatisation conditions. To 100 μl of each cystine standard were added 200 μl of 200 μ*M* homocysteic acid (internal standard), 500 μl of 70 m*M* perchloric acid (containing varying concentrations of MCE ranging from 2.5 m*M* to 125 m*M*), 200 μl of 3 *M* sodium hydroxide. After mixing, 200 μl of a solution of iodoacetic acid of varying concentrations ranging from 10 m*M* to 100 m*M* was added. To 200 μl of this solution were added 10 μl of OPA—MCE reagent and immediately 20 μl were injected onto the column using a filled-loop technique.

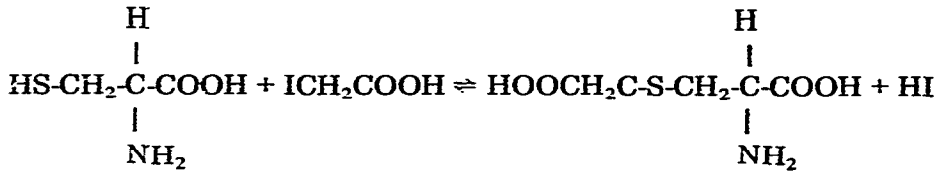
The procedure used when serum or urine was assayed was as follows. To 100 μl of serum or urine were added 200 μl of 200 μ*M* homocysteic acid (internal standard) and 500 μl of protein precipitant (25 m*M* MCE in 70 m*M* perchloric acid solution). After centrifugation at 12,000 *g* for 2 min, 400 μl of the supernatant were added to 100 μl of 3 *M* sodium hydroxide. To 200 μl of this solution were added 100 μl of OPA—MCE reagent and 20 μl immediately injected onto the column using a filled-loop technique.

Quantitation

Peaks on the chromatograms were identified by their retention times and analyte concentrations calculated by proportions using peak area ratio with the internal standard.

RESULTS AND DISCUSSION

The measurement of cystine in physiological samples is important diagnostically in aminoacidopathies. When cystine is reacted with OPA—MCE reagent, because of the reducing conditions, the derivative produced is that of cysteine. The reason cysteine, compared with other primary amines, yields a weakly fluorescent OPA—MCE derivative, although not fully understood, does depend on the presence of sulphydryl groups on the cysteine molecule. By blocking the sulphydryl groups with iodoacetic acid the amino acid forms a strongly fluorescent isoindole product.



Cysteine Iodoacetic acid Cysteine derivative

Initially an excess of iodoacetic acid (100 m*M*) was employed to find the

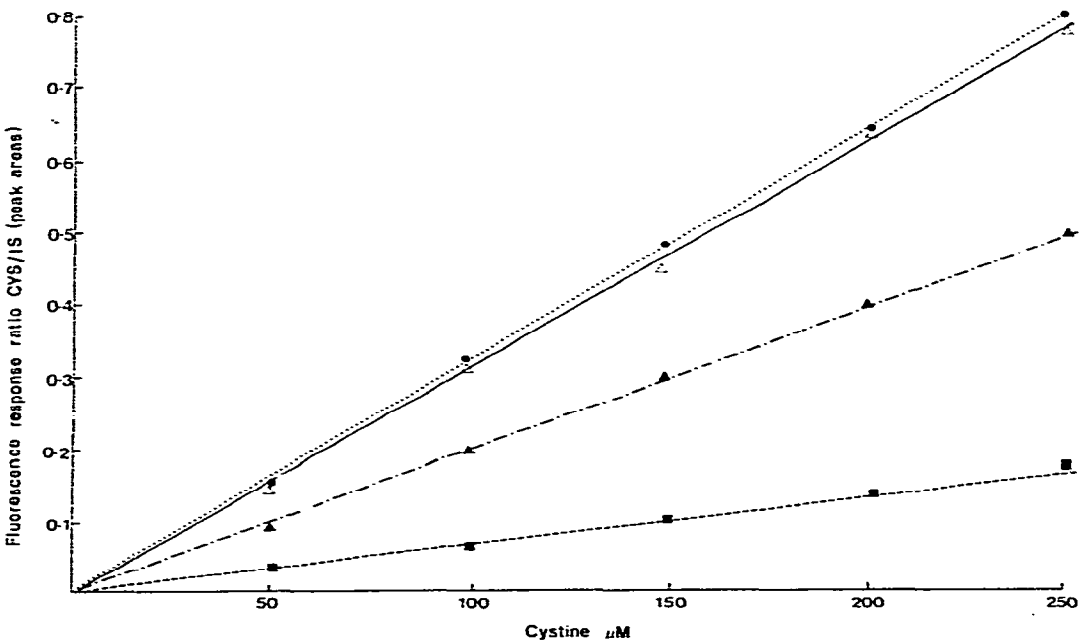


Fig. 1. The effect of varying concentrations of iodoacetic acid and cystine on the relative fluorescence of the cysteine OPA—MCE (CYS) derivative and internal standard (IS). Each point represents a mean of duplicate injections. The derivatisation procedure was performed as described in the text with the following concentrations of iodoacetic acid: •. . . . . •, 100 m*M*; △——△, 50 m*M*; ▲ • — • — ▲, 25 m*M*; ■ — — — ■, 10 m*M*.

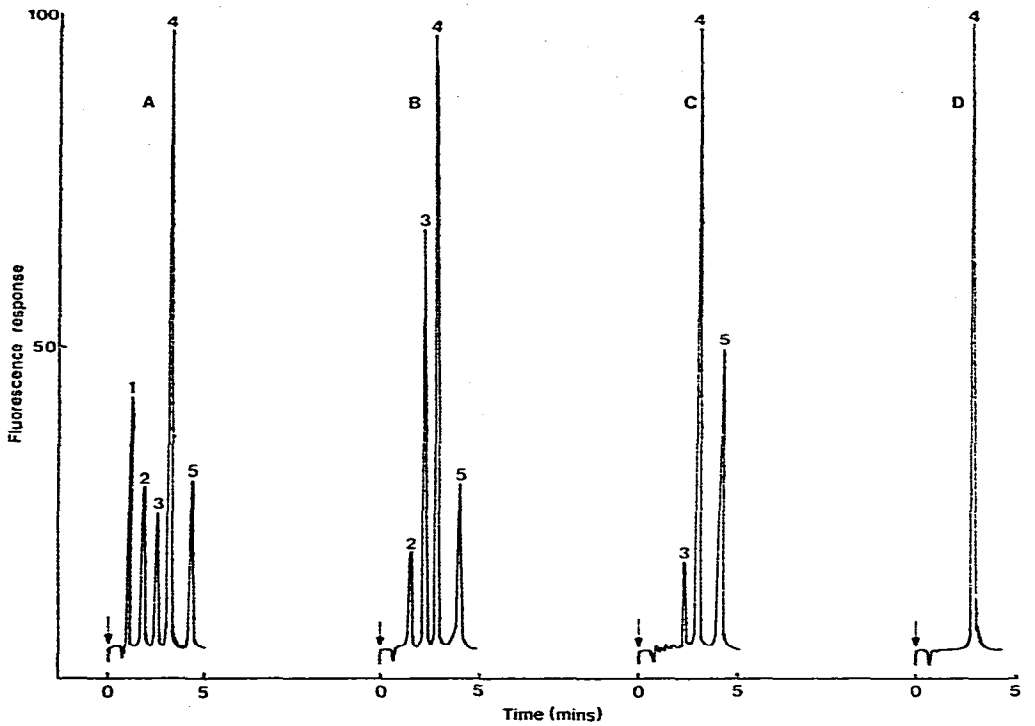


Fig. 2. Chromatograms of the following samples after derivatisation using the method described in the text: A, 100 μM standard solution of each amino acid in water; B, plasma with a cystine concentration estimated to be 99 μM; C, urine with a cystine concentration estimated to be 178 μM; D, internal standard, homocysteic acid. Peaks: 1, phosphoserine; 2, aspartic acid; 3, glutamic acid; 4, internal standard; 5, cystine. Arrow indicates the time of injection.

optimal MCE concentration required for the reduction of cystine. With the dilutions employed this was established as 25 mM MCE in 70 mM perchloric acid. Fig. 1 shows the effect of increasing concentrations of iodoacetic acid on the fluorimetric response of the cysteine derivative. Maximum fluorescence was obtained using 50 mM iodoacetic acid, and the method was linear up to 250 μM cystine.

Fig. 2 shows the chromatograms obtained with serum, urine and a standard cystine solution. The mean cystine concentration of a serum sample injected 20 times was found to be 106 μM with a standard deviation of ± 3.6 μM (coefficient of variation ± 3.4%).

The use of different reagents such as iodoacetamide and acrylonitrile, to block the sulphydryl groups, also enables the formation of different cysteine OPA—MCE derivatives to suit other chromatographic conditions. The fluorescence response of the cysteine OPA—MCE derivative described is linear with different concentrations of cystine allowing quantitative, precise and rapid estimations using this technique.

REFERENCES

1 M. Roth, Anal. Chem., 43 (1971) 880.
2 J.R. Benson and P.E. Hare, Proc. Nat. Acad. Sci. U.S., 72 (1975) 619.
3 [illegible] Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667.
4 S.S. Simons and D.F. Johnson, J. Org. Chem., 43 (1978) 2886.