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Journal of Chromatography B, 712 (1998) 51–58

JOURNAL OF CHROMATOGRAPHY B

Rapid analysis of low levels of indolyl-3-acryloylglycine in human urine by high-performance liquid chromatography

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Received 24 November 1997; received in revised form 10 March 1998; accepted 17 March 1998

Abstract

There have been several reports of increased levels of excretion of indolyl-3-acryloylglycine (IAcrGly) in human urine in a number of disease states. However, the metabolic source of this compound is still not clear and there is the possibility of more than one mechanism for IAcrGly production. There was therefore a need for a rapid, low limit of quantitation assay for IAcrGly to enable further study in this area. In the assay described here, these analytical requirements were addressed by utilising a solid-phase extraction method for sample clean-up, reversed-phase LC with an on-column focusing method of sample introduction and UV absorbance detection at 326 nm. The limit of quantitation of this method was 26.2 ng ml^{-1}. It was also noted that IAcrGly undergoes isomerisation when exposed to light and that this process is reversible.

Keywords: Indolyl-3-acryloylglycine

1. Introduction

For many years, there has been interest in the levels of indolyl-3-acryloylglycine (IAcrGly, Fig. 1) excreted in human urine and the physiological role of this compound in various disease states. For example, increased levels of IAcrGly have been reported in Hartnup disease [1] and skin disease [2]. In the widest survey conducted to date, excretion of IAcrGly has been reported in 71% of the normal population [3] but the thin-layer chromatography (TLC) method used was relatively insensitive and this figure may therefore be an underestimate of the proportion of the normal population which excrete this compound. Data on the normal levels of excretion of IAcrGly in human urine are, therefore, limited and the biochemical reasons for elevated levels of IAcrGly are still unclear [4]. One proposed method is that tryptophan is converted to indole-3-propionic acid by intestinal flora in the gut and that the indole-3-propionic acid is then converted to indole-3-acrylic acid and its glycine conjugate (IAcrGly) in the liver or kidney [5]. However, an alternative and possibly minor mechanism which does not involve the action of gut flora has been suggested by Marklova et al. [6].

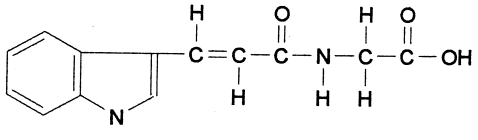

Fig. 1. Indolyl-3-acryloylglycine.

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0378-4347/98/$19.00
PII: S0378-4347(98)00153-4

There is, therefore, the need for further study in this area to obtain further information on the extent and mechanism of IAcrGly excretion in the normal population and to gain an understanding of the reasons behind unusual levels of IAcrGly excretion in abnormal physiological conditions.

It was expected that in the course of such a study that there would be a need for a low limit of quantitation assay for IAcrGly in urine. The earliest paper chromatography [7] and TLC based methods [5,8] for IAcrGly analysis lacked sensitivity. These methods were subsequently improved by Marklova and Hais using Sephadex gels [9] and more recently, by reversed-phase liquid chromatography (LC) [4], which has been the most simple method and lowest limit of quantitation (LOQ) reported to date (calibration range 1 to 100 μg ml^{-1}). However, there remains the possibility that a still lower LOQ will be required for metabolic studies, particularly if there are minor routes involved in IAcrGly production. Also in the method described by Marklova and Fojtaskova [4], losses of IAcrGly may occur as a result of a precipitation step with zinc sulphate and sodium hydroxide. A simplified extraction procedure which avoids the use of such a precipitation step was therefore desirable to eliminate this source of sample loss and to reduce sample preparation time.

In this paper, an alternative assay is described which is faster, more specific to IAcrGly and which provides a lower LOQ for this compound than has until now been possible.

2. Experimental

2.1. Reagents and materials

IAcrGly was synthesised via esterification of indole-3-acrylic acid Sigma (Poole, UK) with equimolar quantities of glycine methyl ester (Sigma) dicyclohexylcarbodiimide and hydoxybenzotriazole (Aldrich, Gillingham, UK) in dichloromethane (BDH, Poole, UK). This was followed by base catalysed hydrolysis of the ester group with aqueous/methanolic lithium hydroxide (Aldrich) to give the carboxylic acid. The basic reaction mixture was the neutralised with glacial acetic acid (Sigma) and the IAcrGly isolated via LC and peak collection using a preparative chromatographic system consisting of a 250×10 mm I.D. Vydac ODS2 (5 μm) column (Phenomenex, Macclesfield, UK) and a mobile phase of acetonitrile–0.1% (v/v) aqueous formic acid (18:72, v/v). Peak purity was greater than 99% area by LC (system 1), following freeze drying of the collected fractions and reconstitution in mobile phase.

Indole-3-acetic acid, indole-3-propionic acid and formic acid (analytical-reagent grade) were supplied by Sigma. Acetonitrile and methanol, Hypersolv (HPLC grade) were supplied by BDH and trifluoroacetic acid (TFA) (99.99%) was supplied by Aldrich. Urine samples, first in the morning, mid stream were collected without preservatives and stored frozen at −20°C until analysis. A standard additions method of calibration was used and a stock of control urine, diluted 50:50 (v/v) with de-ionised water was used for all standard additions (standard A). A standard solution of IAcrGly (210 μg ml^{-1}) was prepared in de-ionised water. This solution was then further diluted to give a range of aqueous IAcrGly calibration standards spanning the range 26.2 ng ml^{-1} to 105 μg ml^{-1}. The stock IAcrGly standard was stored for up to four weeks and the dilute standards were prepared weekly and stored at −20°C.

2.2. Chromatography

Chromatographic equipment consisted of Shimadzu LC10-AD and LC 6-AD solvent delivery modules with Shimadzu SP6-AD UV–Vis detector and Shimadzu integrator all from Dyson Instruments (Houghton-le-Spring, UK). Sample injections were made with SGE gas tight syringes via a Rheodyne 7125 valve (Anachem, Luton, UK). Analytical columns were supplied by Hichrom (Reading, UK), except the Vydac column (Phenomenex). Throughout the course of this study, the following LC systems were used. In each system the detection wavelength was 326 nm and the column temperature was ambient (25°C to 28°C).

2.2.1. HPLC system 1

Column: 150×4.6 mm I.D. Nucleosil C_{18} (5 μm) with a 10×4.6 mm I.D. Nucleosil C_{18} (10 μm) guard column. Mobile phase: methanol–0.1% (v/v) aqueous formic acid (40:60, v/v), flow-rate 1.5 ml min^{-1}.

2.2.2. HPLC system 2

Column: 150×4.6 mm I.D. Nucleosil C_{18} (5 μm) with a 10×4.6 mm I.D. Nucleosil C_{18} (10 μm) guard column. Mobile phase: acetonitrile–0.1% (v/v) aqueous TFA (18:72, v/v), flow-rate 1.5 ml min^{-1}.

2.2.3. HPLC system 3

Column: 25×4.6 mm I.D. Vydac ODS2 (5 μm). Mobile phase: isocratic, acetonitrile–0.1% (v/v) aqueous TFA (5:95, v/v) for 8 min, followed by a gradient of 5–50% (v/v) acetonitrile–0.1% (v/v) aqueous TFA over 8–40 min, flow-rate: 2.0 ml min^{-1}.

2.2.4. HPLC system 4

Column: 150×4.6 mm I.D. Nucleosil aminopropyl (5 μm) with a mobile phase of acetonitrile–10 m*M* ammonium acetate (10:90, v/v), flow-rate 1.0 ml min^{-1}.

2.3. Solid-phase extraction (SPE) and IAcrGly recovery

In order to determine the optimum conditions for SPE, studies were first undertaken to determine the recovery IAcrGly from aqueous solution. This study was conducted with C_2, C_8, C_{18}, phenyl and cyanopropyl phases (1 ml×100 mg Bond Elut SPE cartridges (Phenomenex) and with a range of eluents of increasing methanol or acetonitrile concentration. Recovery was determined by comparison of the detector response obtained following injection (20 μl) of a standard solution of IAcrGly onto the LC (system 1), with the responses obtained from LC analysis of the SPE effluents. Having optimised SPE conditions with aqueous samples, the effectiveness of each phase for sample clean-up was assessed by test extraction of urine. Method validation then progressed with the use of C_8 SPE cartridges.

2.3.1. Solid-phase extraction

To 0.1 ml samples of test urine and/or aqueous IAcrGly calibration standards was added 0.1 ml of aqueous control urine standard (standard A) and 0.1 ml of 0.1% (v/v) aqueous acetic acid. The samples were then vortex mixed and applied to 1 ml×100 mg, C_8 Bond Elut SPE cartridges which had been pre-conditioned with 1 ml of methanol followed by 1 ml of 0.1% (v/v) aqueous acetic acid. The samples were then washed with 3×1 ml of methanol–0.1% (v/v) aqueous acetic acid (5:95, v/v) and then eluted with 0.75 ml of methanol–0.1% (v/v) triethylamine (TEA) (40:60, v/v). The samples were then acidified and diluted with 0.25 ml of 0.1% (v/v) aqueous acetic acid prior to injection onto the LC column. For samples in the range 5–105 μg ml^{-1}, 50 μl of the extracts were injected and below this range the injection volume was 200 μl (LC system 1).

2.4. Selectivity

To determine assay selectivity, the IAcrGly peak fraction resulting from the LC (system 1) of an extract of 1 ml of urine from a person with autism was collected. A 20-μl volume of this solution was injected onto LC systems 2–4.

Molecular mass determination of the major extracted component was obtained from a freeze–dried peak fraction using a triple quadrupole LC–MS–MS mass spectrometer (supplied by Perkin-Elmer Sciex Instruments, Warrington, UK) with atmospheric pressure chemical ionisation (APCI). For these experiments a 15 cm×2.1 mm I.D. Nucleosil ODS2 (5 μm) column (Capital HPLC, Broxburn, UK) was used with a mobile phase of acetonitrile–0.1% (v/v) aqueous formic acid (18:82, v/v) and flow-rate of 400 μl min^{-1}. Injections were made in mobile phase. In addition nuclear magnetic resonance (NMR) (Jeol GSX270) spectra (D_20, CD_3CN and two-dimensional NMR) were obtained and these data were compared with those obtained with a synthetic sample of IAcrGly.

In addition, indole-3-acetic acid, indole-3-propionic acid and indole-3-acrylic acid [1 mg ml^{-1} in methanol–0.1% (v/v) aqueous formic acid (40:60, v/v)] were injected onto LC system 1 to determine whether the presence of these compounds in urine would be likely to produce an analytical interference.

2.5. Assay performance

Accuracy, precision, linearity and LOQ were established by replicate analyses of IAcrGly spiked samples of urine spanning a range of concentrations

(0.0262 μg ml^{-1}, 1.05 μg ml^{-1} and 105 μg ml^{-1}) on separate days.

2.6. Effect of light on IAcrGly

The collected IAcrGly peak fraction following LC (system 4) of an extract of 10 ml of a sample of urine from a person with autism was freeze–dried and reconstituted in 1 ml acetonitrile–0.1% (v/v) aqueous TFA (5:95, v/v). A 20-μl volume of this sample was injected onto LC system 2. The remaining sample was irradiated for 30 min under a broad spectrum (254–366 nm, 50 Hz) UV lamp (UVPINC, San Gabriel, CA, USA) before injection of a further 20 μl aliquot. The irradiated sample was then stored at 4°C in the dark prior to further sampling and LC analysis over a range of time periods up to 18 h.

3. Results and discussion

3.1. Solid-phase extraction and IAcrGly recovery

C_8 SPE cartridges with methanolic eluent were selected for assay development and validation because this system provided the cleanest extracts and optimum recovery. IAcrGly recovery from aqueous solution with C_8 SPE cartridges is shown in Fig. 2. Recovery increased up to around 90% with increasing methanol content in the SPE eluent and near maximum recovery (88%) was obtained with methanol–0.1% (v/v) TEA (40:60, v/v). This solvent system was therefore identified as the optimum eluent since there was little additional recovery obtained with greater methanol concentrations and it was desired to minimise the concentration of organic solvent in the eluant to allow direct injection onto the LC column. Recovery from spiked urine samples was similar to the results obtained from the aqueous samples.

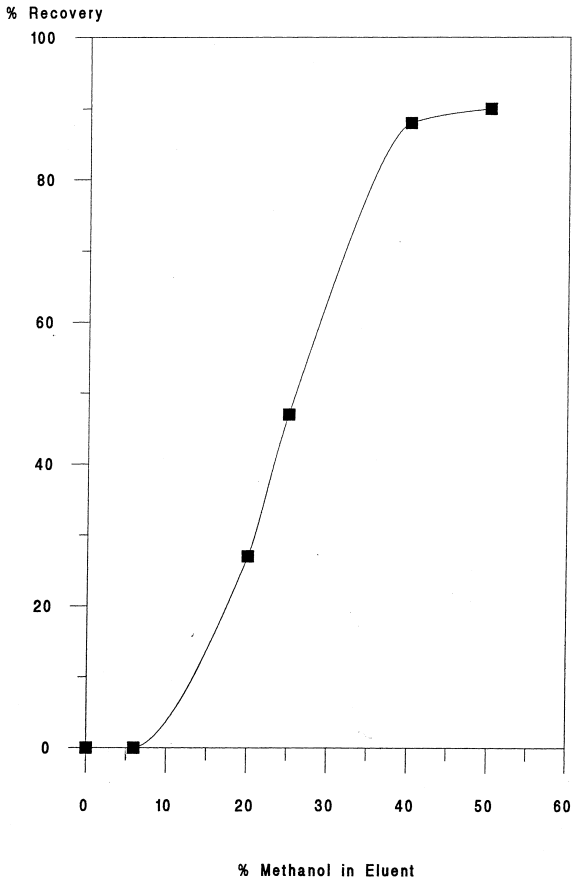

Fig. 2. Recovery of IAcrGly from C_8 SPE cartridges with increasing concentration of methanol in the SPE eluent. Recovery was determined by comparison of the peak areas obtained by LC for extracted samples with the area obtained for a non-extracted sample. See Section 2.3.1 for details.

3.2. Selectivity

The method was found to be selective for IAcrGly, since a collected peak fraction was determined to be one peak on the four chromatographic systems tested. Peak identification was confirmed by co-elution of a synthesised sample of IAcrGly on chromatographic systems 1–4 [system 1 retention factor (k)=4.2; system 2 k=6.9; system 3 k=13.3; system 4 k=7.0] and by the UV spectra of an isolated peak fraction and synthesised IAcrGly [maxima 220, 270 and 324 nm; sample solvent acetonitrile–0.1% (v/v) aqueous TFA (18:72, v/v)].

The mass spectrum resulting from LC–APCI-MS of the IAcrGly sample extracted from urine exhibited a weak M+1 ion (m/z 245) in positive ion mode, and a high abundance M−1 ion in negative ion mode, indicating a molecular mass of 244. The ^1H NMR spectrum of the extracted and synthetic IAcrGly in D_2O and CD_3CN, indicated the presence of an indole nucleus (indole C2-H, doublet at d7.5, J3Hz)

linked through a trans acrylic acid (aCH=, d6.48, J15.8Hz; bCH=, d7.69, J15.8) to a glycine unit (CH_2, d3.91, J5.6Hz).

In addition, aqueous solutions of the collected LC fraction and the synthetic sample turned red on addition of 0.1 *M* HCl. This colour change in acid solution is a modification of the ''uro-rosein'' reaction [10].

IAcrGly is particularly susceptible to degradation by strong inorganic acids and is also degraded albeit more slowly in 0.1% (v/v) aqueous TFA. However, samples were more stable in aqueous solutions of weaker organic acids such as formic or acetic acid. For this reason the chromatographic system for the assay of IAcrGly (system 1) made use of formic acid as a pH modifier in preference to aqueous TFA, to avoid the possibility of degradation of the sample on the column. Samples were found to be stable for at least one week at −20°C after acidification with formic acid and for at least 24 h at room temperature when protected from light.

A typical chromatogram (system 1) of an extract of urine from a control subject is shown in Fig. 3. No chromatographic interference occurred with indole-3-acrylic acid or indole-3-propionic acid which are precursors to IAcrGly in-vivo [5]. Indole-3-acetic acid co-eluted with IAcrGly (k=4.2 min), but indole-3-acetic has no absorbance at 326 nm which was the detection wavelength used in this study.

It was not possible to obtain a sample of urine which did not contain detectable levels of IAcrGly and therefore a standard additions method was used to calibrate the assay. To obtain the lowest quantitation limits (0.0262 μg ml^{-1}) it was necessary to inject 200 μl of sample. However, for samples in the upper part of the assay range, an injection of not more than 50 μl was necessary to avoid exceeding the dynamic range of the detector. Two separate standard lines were therefore used, 0.0262 to 5.25 μg ml^{-1} and 5.25 to 105 μg ml^{-1}. Typical regression equations for these lines were $y=9907+9.4x$ ($R^2=0.999$) and $y=-2668+3590x$ ($R^2=0.999$), respectively.

3.3. Assay performance

Accuracy and precision were assessed by extracting and injecting three replicate samples of spiked

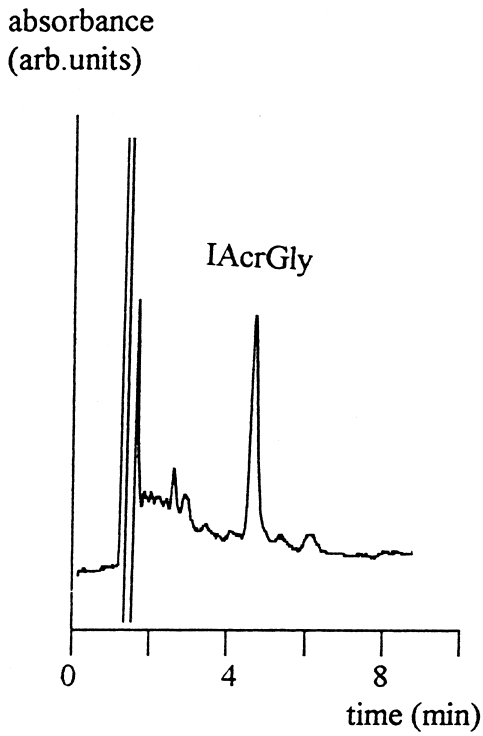

Fig. 3. Chromatography following injection of an extract of urine from a control subject (equivalent to 498 ng ml^{-1} of IAcrGly in urine). Column: 150×4.6 mm I.D. Nucleosil C_{18} (5 μm). Mobile phase: methanol–0.1% (v/v) aqueous formic acid (40:60, v/v), flow-rate 1.5 ml min^{-1}. Injection volume: 200 μl, detection 326 nm.

standards on two separate days. The determined mean concentrations (Table 1) were within 8% of the nominal values in each case and the relative standard deviations (R.S.D.s) for the 1.05 and 105 μg ml^{-1} standards were 12% and 1.4%, respectively. An R.S.D. of 19.8% was obtained with the 0.0262 μg ml^{-1} sample which was accepted as the limit of quantitation (LOQ) of the assay. The limit of detection (LOD) based on the definition of three-times the signal-to-noise ratio was 500 pg ml^{-1}. The large difference between the LOQ and LOD reflects a disadvantage of the standard addition method of analysis. The detector response to the lowest spiked standard was less than 5% of that due to the IAcrGly which was endogenous in the standard. Therefore at the lower end of the standard curve range the LOQ was limited by the ability of the system to detect small increases in the signal against a large back-

Table 1
Inter-day precision of determined IAcrGly concentrations following the assay of extracted urine standards

	Concentration (μg ml^{-1})		
	0.0262[a]	1.05[a]	105[a]
Day 1	0.0246	1.24	105.5
	0.0307	1.17	102.3
	0.0290	1.04	106.2
Day 2	0.0189	0.94	106.5
	0.0319	0.94	105.7
	0.0342	1.17	105.7
Mean	0.0282	1.08	105.3
S.D.	0.00559	0.13	1.52
R.S.D. (%)	19.8	12.0	1.4
Accuracy (%)	7.6	2.9	0.3

See Section 2 for details.
[a] Nominal concentration.

ground. This factor would be less problematic with the more usual spiked blank matrix calibration methods which determine increases in detector signal against a blank background.

However, screening of urine from 49 control subjects failed to produce a sample which did not contain IAcrGly and which could therefore be used as a blank. This observation indicated that the described method would have more than adequate LOQ for a survey of IAcrGly levels in the normal population. Since such a study would involve the analysis of many samples, this method also had the advantage of reduced chromatographic run time and therefore greater sample throughput compared to previously reported methods. In addition, a reduction in the sample preparation time resulted from the elimination of drying and reconstitution steps after SPE. This was made possible by employing an on-column focusing method of sample injection onto the analytical column and was accomplished by adding a small quantity of aqueous acetic acid diluent (0.25 ml) to the SPE eluant prior to sample injection. The concentration of methanol in the injection solvent was therefore reduced to 30% (v/v) compared to 40% (v/v) methanol in the mobile phase. Thus, when the sample was injected, focusing of the analyte band occurred on the top of the column [11]. Preliminary studies with on-column focusing has shown that up to 500 μl of the above extracts may be injected while still maintaining resolution of the analyte from co-extracted endogenous compounds and there is therefore potential for achieving still lower quantitation limits by increasing the injection volume.

3.4. Effect of light on IAcrGly

An interesting observation resulting from this study concerned the conversion of IAcrGly from one isomer to another after exposure to light. This phenomenon was first noted when samples of IAcrGly which had originally contained one peak, produced a second, later eluting peak after storage in clear glass vials under the laboratory strip lighting. After the samples were returned to the dark, this second peak disappeared on re-analysis of the samples.

This effect was studied in more detail by exposing a sample of IAcrGly to UV radiation for 30 min and then determining the peak heights of IAcrGly and the second component over a period of time after returning the sample to darkness.

The chromatograms obtained from this sample before and immediately after irradiation are shown in Fig. 4. Before irradiation, the IAcrGly sample produced only one peak at the expected retention time of IAcrGly. However, after the irradiation period, a second peak was evident with concomitant reduction of the height of the first peak. Upon further LC analysis, after returning the sample to darkness, the height of the second peak diminished and the height of the first peak returned to near its original value (Fig. 5).

This conversion between the second LC peak and the IAcrGly peak was further established by collecting the peak fraction from this second component. After storage overnight at 4°C in the dark followed by re-injection of this sample onto the LC system, it was found that this second component was no longer detectable and the sample contained only IAcrGly. The structure of this second sample component is not certain. However, since it appears to arise as a result of a reversible photolysis of IAcrGly, it is possible that it results from *cis*/*trans* isomerisation. This form of isomerisation has been proposed in the past to explain the appearance of a second component during the analysis of indole-3-acrylic acid, a precursor to the in vivo production of IAcrGly [12].

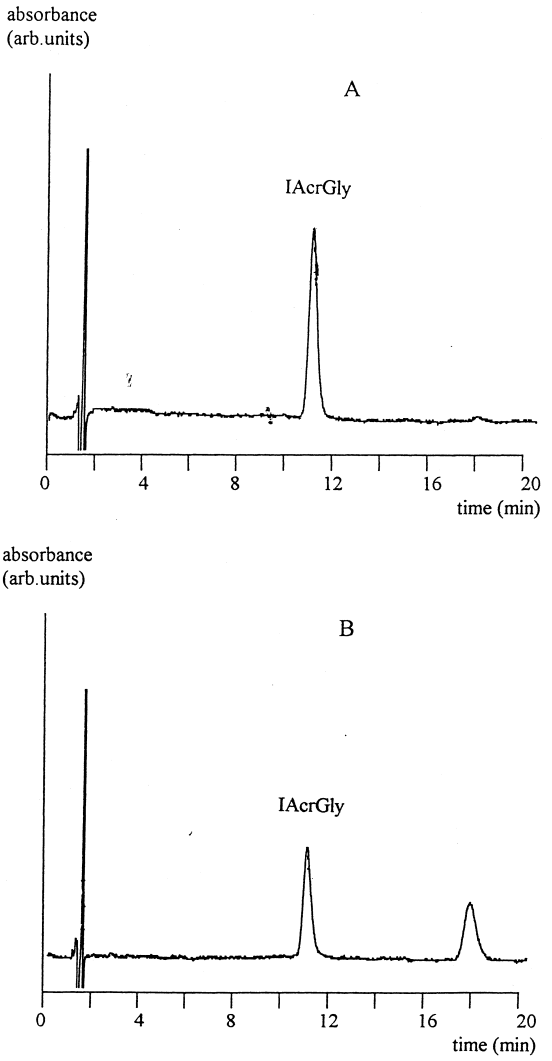

Fig. 4. Chromatography following injection of a solution of IAcrGly in acetonitrile–0.1% (v/v) aqueous TFA (5:95, v/v) before (A) and after (B) irradiation of the sample at 255–366 nm for 30 min. Approximate IAcrGly on-column loadings: 70 ng (A) and 40 ng (B). Chromatographic conditions: column: 150×4.6 mm I.D. Nucleosil C_{18} (5 μm). Mobile phase: acetonitrile–0.1% (v/v) aqueous TFA (18:72, v/v), flow-rate 1.5 ml min^{-1}. Injection volume: 20 μl, detection 326 nm.

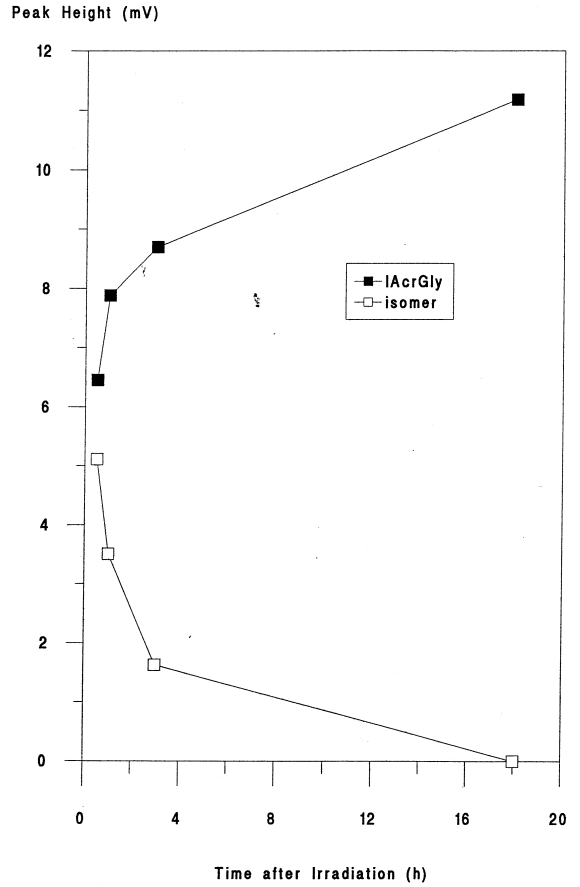

Fig. 5. Effect on the peak height of IAcrGly and the isomer peak over time, following chromatography of an irradiated sample of IAcrGly which had been returned to the dark. See Section 2.6 for details.

4. Conclusions

A new assay for IAcrGly in human urine has been developed which allows faster sample throughput compared to previous methods and which offers the additional advantage of providing a lower LOQ. Early indications are that this assay will have sufficient sensitivity to determine IAcrGly levels in a planned study of excretion levels of this compound in the normal population.

In addition, evidence has been obtained which shows that IAcrGly in solution exists as a mixture of isomers when exposed to light and that the conversion from one isomer to the other is reversible.

Acknowledgements

The authors would like to express their gratitude to the University of Sunderland for providing the funding and facilities for this research and to Paul

Groundwater and David Bendell for their help in obtaining and interpreting the NMR data. We would also like to express our thanks to Terry Noctor and Darren Thomas at Corning Hazelton for obtaining the MS data and to Drug Development (Scotland) Ltd. for supplying control urine.

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