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# Identification of indolyl-3-acryloylglycine in the urine of people with autism

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**Abstract**

HPLC analysis of the urine of autistic subjects indicated the presence of an unidentified component in greatly increased concentrations. We have reported the isolation of this component by HPLC and its identification. Mass spectrometry, NMR and UV spectroscopy identified the peak as corresponding to indolyl-3-acryloylglycine (IAG, **3**), and this has been confirmed by an independent synthesis.

## Introduction

Autism is a lifelong pervasive developmental disorder that manifests as problems with communication, social skills and imagination. It affects 80000 people in the UK (data from the National Autistic Society, London 1997) and, at present, can only be diagnosed by reference to behavioural and psychological characteristics. For many years there has been interest in the analysis of body fluids from people with autism in the search for unusual correlates which could be used as markers for the disorder, and which would provide evidence for a physiological dysfunction (Panksepp 1979; Gillberg 1988; Adrien et al 1989; Knivsberg et al 1990; Shattock et al 1990). As a result of an ongoing programme in this laboratory, screening the urine from people with autism by high-performance liquid chromatography (HPLC), it was noted that there was a particular peak present in all the chromatograms from the sample population (Mills et al 1998). This peak was often present in greatly increased concentrations, based on peak height and area by HPLC, compared with those found in controls. We have reported the isolation and structural elucidation of this compound.

## Materials and Methods

### Materials

Chemicals and solvents were obtained from Aldrich-Sigma-Fluka unless indicated otherwise. UV spectroscopy was carried out on a Shimadzu SP6-AD UV-vis detector, which was set to 326 nm for HPLC detection. Melting points were determined on a Gallenkamp apparatus and are uncorrected. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a BRUKER AVANCE 300 NMR spectrometer at 300

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and 75.5 MHz, respectively, or on a JEOL GSX NMR spectrometer at 270 MHz for proton spectra. Tetramethylsilane was used as an internal standard and coupling constants were reported in Hz. The molecular weight was determined using a triple quadruple LC/MS/MS PERKIN-ELMER mass spectrometer (Sciex Instruments, UK) in LC-APCI mode. Accurate mass determinations were carried out on a BRUKER APEX II FTMS (electrospray ionization). Preparative reverse-phase TLC was carried out on Whatman pre-coated glass plates, KC18F 60Å $SiO_2$, with C18 bonded phase at a loading of 50-mg sample per plate. Solid-phase extraction was conducted using $C_2$, $C_8$, and $C_{18}$ phenyl and cyanopropyl phases on 1 mL × 100 mg Bond Elut SPE cartridges from Phenomenex. Several different HPLC columns from Phenomenex and mobile phase systems were used to isolate, purify and characterize the compound of interest as indolyl-3-acryloylglycine (IAG). HPLC system 1: 150 × 4.6 mm i.d. Nucleosil aminopropyl (5 $\mu$m), mobile phase: acetonitrile : 5 mM ammonium acetate (95 : 5, v/v), flow rate: 1.0 mL $min^{-1}$. HPLC system 2: 150 × 4.6 mm i.d. Nucleosil $C_{18}$ (5 $\mu$m) with a 10 × 4.6 mm i.d. Nucleosil (10 $\mu$m) guard column, mobile phase: methanol : 0.1% (v/v) aqueous formic acid (40 : 60, v/v), flow rate: 1.5 mL $min^{-1}$. HPLC system 3 for LC-MS: 150 mm × 2.1 mm i.d. (5 $\mu$m) Nucleosil ODS2 column, mobile phase: 0.1% v/v aqueous formic acid : acetonitrile (82 : 18, v/v), flow rate: 400 $\mu$L $min^{-1}$.

### Isolation of indolyl-3-acryloylglycine from biological samples for spectroscopy

Urine was obtained from selected volunteers with autism who were able to give informed consent. It was passed through three separate solid-phase extraction Bond Elut cartridges and lyophilized before reconstitution into 5% aqueous acetonitrile (0.75 mL) and injection onto HPLC system 1. The compound that gave rise to the peak of interest was then collected and lyophilized. Once dry, the sample was reconstituted in 5% v/v acetonitrile in 0.1% aqueous formic acid (0.5 mL) and microcentrifuged for 10 min. The resulting supernatant was then injected onto HPLC system 2 and the peak of interest collected. Due to the instability of the compound in a strongly acidic environment, the sample was buffered with ammonium acetate before a second lyophilization. Any traces of ammonium acetate were removed via a final solid-phase extraction procedure (using a $C_{18}$ Bond Elut cartridge) and the product was eluted with acetonitrile to give indolyl-3-acryloylglycine (1.5 mg); $m/z$ (LC-APCI MS) 245 (M + 1) in positive ion mode, 243 (M − 1) in negative ion mode; UV (0.1% v/v aqueous formic acid or 0.1 M NaOH) 226, 277 and 326 nm; $\delta_H$ ($CD_3CN$) 3.91 (2H, d, $J$ 5.6, $CH_2$), 6.49 (1H, d, $J$ 15.8, $=CH_\alpha$), 6.69 (1H, br s, NHCO), 7.14 (2H, m, H-5 and H-6), 7.40 (1H, d, $J$ 5.9, H-7), 7.52 (1H, d, $J$ 3, H-2), 7.69 (1H, d, $J$ 15.8, $=CH_\beta$), 7.85 (1H, d, $J$ 6.5, H-4), 11.80 (1H, br s, H-1).

### Molecular weight determination

The major component extracted from the urine samples was freeze-dried, then reconstituted in acetonitrile : 0.1% (v/v) aqueous formic acid (18 : 82, v/v) and injected into the mass spectrometer (Mills et al 1998).

### Synthesis of indolyl-3-acryloylglycine methyl ester 2

Indolyl-3-acrylic acid **1** (0.5 g, 2.67 mmol), 2-chloro-1-methylpyridinium iodide (0.819 g, 3.2 mmol), tri-*n*-butylamine (1.98 g, 2.55 mmol) and glycine methyl ester hydrochloride (0.339 g, 2.7 mmol) were dissolved in dry dichloromethane (12 $cm^3$) and the reaction mixture was stirred for 24 h. The organic phase was washed with 1 M aq. HCl (20 $cm^3$), followed by water (20 $cm^3$), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by flash chromatography on silica, eluting with ethyl acetate/petrol 40–60 (50 : 50–100 : 0), followed by recrystallization from chloroform, gave *E*-indolyl-3-acryloylglycine methyl ester **2** as white needle-like crystals (0.49 g, 71%); mp 126–127°C [lit. (Inhoffen et al 1963) mp 161–163°C; isomer not specified]. (Found: $MH^+$ 259.1077. Calc. for $C_{14}H_{15}N_2O_3$: *MH*, 259.1076. Found: $MNa^+$, 281.0897. Calc. for $C_{14}H_{14}N_2O_3Na$: *MNa*, 281.0896.) $\nu_{max}$ 3345 (NH), 1735 (ester C=O), 1660 (amide C=O), and 1604 (C=C) $cm^{-1}$; $\delta_H$(300 MHz, $CDCl_3$) 3.81 (3H, s, $CO_2CH_3$), 4.22 (2H, d, $J$ 5.2, $CH_2NH$), 6.12 (1H, br t, NHCO), 6.50 (1H, d, $J$15.6 $=CH_\alpha$), 7.26 (2H, m, H-5 and H-6), 7.43 (1H, dd, $J$ 6.9 and 1.9, H-7), 7.46 (1H, d, $J$ 2.7, H-2), 7.89 (1H, d, $J$ 15.6, $=CH_\beta$), 7.90 (1H, dd, $J$ 5.6 and 2.3, H-4), 8.78 (1H, br s, H-1); $\delta_C$ (75.5 MHz, $CDCl_3$) 41.6 ($CH_3$), 41.7 ($CH_2$), 113.0 (quat.), 113.1 (CH), 116.4 (CH), 120.7 (CH), 121.2 (CH), 123.0 (CH), 125.9 (quat.), 131.0 (CH), 134.5 (CH), 138.3 (quat.), 167.6 (C=O), 171.5 (C=O).

### Synthesis of indolyl-3-acryloylglycine (3)

Indolyl-3-acryloylglycine methyl ester **2** (100 mg, 0.36 mmol) was dissolved in phosphate buffer (20 mM, pH 8, 18 $cm^3$) and acetone (2 $cm^3$) and pig liver esterase

(2000 U) was added. The reaction mixture was stirred for five days and filtered before freeze-drying. Purification by reverse phase TLC, eluting with acetonitrile: water (1:3), gave indolyl-3-acryloylglycine (**3**) as a colourless semi-solid (35 mg, 37%). (Found: $MNa^+$, 267.0741. Calc. for $C_{13}H_{12}N_2O_3Na$: *MNa*, 267.0740.) $\lambda_{max}$ (0.02 M phosphate-borate buffer with 0.1 M sodium cholate, pH 9) 232, 280, and 330 nm; $\delta_H$ (300 MHz, $d_6$-DMSO) 3.55 (2H, d, *J* 4.7, $CH_2$), 6.82 (1H, d, *J* 15.8, $=CH_\alpha$), 7.15 (2H, m, H-5 and H-6), 7.44 (1H, d, *J* 7.7, H-7), 7.55 (1H, t, *J* 5.0, NHCO), 7.57 (1H, d, *J* 15.8, $=CH_\beta$), 7.71 (1H, s, H-2), 8.02 (1H, d, *J* 7.3, H-4), 11.80 (1H, br s, H-1); $\delta_C$ (75.5 MHz, $d_6$-DMSO) 45.2 ($CH_2$), 113.0 (CH), 113.2 (CH), 118.0 (quat.), 118.5 (CH), 120.9 (CH), 121.1 (CH), 122.5 (CH), 122.8 (CH), 125.9 (quat.), 133.0 (quat.), 166.4 (C=O), 174.5 (C=O).

## Results and Discussion

Mass spectrometry, $^1$H NMR and UV spectroscopy were used to identify the compound responsible for this peak. The mass spectrum, using LC-MS (atmospheric pressure chemical ionization), indicated a molecular mass of 244. UV spectra were recorded at high and low pH to investigate the presence of acidic and/or basic groups, via bathochromic or hypsochromic shifts. At low pH, the absorptions in the UV spectrum, at 226, 277 and 326 nm, were suggestive of a heteroaromatic system with further conjugation giving rise to a shift to longer wavelength. Treatment with NaOH made no difference to the UV spectrum. Finally, the $^1$H NMR of the compound, recorded in $CD_3CN$ as this had been the solvent used in the chromatography, indicated the presence of an indole nucleus (indole C2-H, doublet at $\delta$7.5, *J* 3 Hz) linked through a *trans* acrylic acid ($\alpha$CH=, $\delta$6.48, *J* 15.8 Hz; $\beta$CH=, $\delta$7.69, *J* 15.8 Hz) to a glycine unit ($CH_2$, $\delta$3.91, *J* 5.6 Hz). The full structure was elucidated using the spectral data obtained and the compound identified as indolyl-3-acryloylglycine (**3**).

To confirm the identity of the compound, we synthesized indolyl-3-acryloylglycine independently. The synthesis was achieved via the Mukaiyama coupling (Bald et al 1975) of indoleacrylic acid (**1**) with glycine methyl ester to give the ester **2**, which yielded the *trans* isomer exclusively, as confirmed by the $^1$H NMR spectrum. Comparison of the melting point of the ester **2** with that reported in the literature (Inhoffen et al 1963) suggested that the isomer prepared was the *cis* isomer. Hydrolysis of the ester group gave the carboxylic acid **3** (Figure 1). This hydrolysis was accomplished using pig liver esterase (E.C. 3.1.1.1) due to the sensitivity of indolyl-3-acryloylglycine (**3**) to acid, precluding either acid-catalysed hydrolysis or acidification after a base-catalysed reaction. Purification by lyophilization, followed by preparative reverse phase TLC gave a sample that was spectroscopically and chromatographically identical to

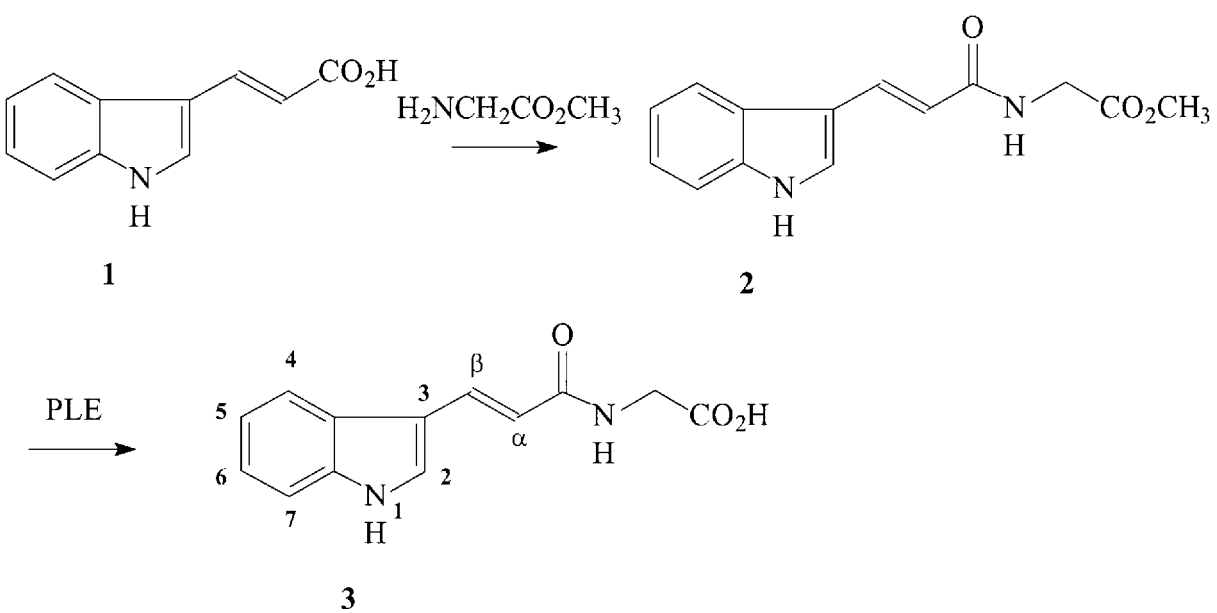


**Figure 1** Independent synthesis of indolyl-3-acryloylglycine (**3**). PLE, pig liver esterase.

that originally obtained from the urine of the subjects with autism. The UV and NMR spectroscopy of synthetic indolyl-3-acryloylglycine were carried out in more conventional spectroscopic solvents than had been used to investigate the structure of the unknown compound.

It is known that indolylacrylic acid (IAcrA) and/or indolyl-3-acryloylglycine levels are elevated in a number of disease states, including Hartnup Disease (Milne et al 1960), light sensitive dermatitis (Kimmig et al 1958) and phenylketonuria (Strohmayer 1964), but levels of indolyl-3-acryloylglycine had not previously been reported as being elevated in autism. Previous reports had shown that 71% of the normal population excreted indolyl-3-acryloylglycine in varying amounts (Mandell & Rubin 1965; Marklová et al 2000). The source of indolyl-3-acryloylglycine in autistic subjects is not clearly understood but has been linked to defects in tryptophan metabolism (Marklová 1999). It is assumed that the indolyl-3-acryloylglycine is a metabolic product from the precursor indolyl-3-acrylic acid, which could be produced by gut flora or by endogenous routes (Smith et al 1968).

## Conclusions

The compound noted as being present in markedly increased amounts in the urine of autistic subjects was isolated by HPLC and was identified as indolyl-3-acryloylglycine using UV and NMR spectroscopy, and MS spectrometry. An independent chemical synthesis of indolyl-3-acryloylglycine confirmed the structure as indolyl-3-acryloylglycine as did comparison of the spectral features with the urine-derived indolyl-3-acryloylglycine.

## References


Adrien, J. L., Barthelemy, C., Lelord, G., Muh, J. P. (1989) Use of bioclinical markers for the assessment and treatment of children with pervasive developmental disorders. *Neuropsychobiology* **22**: 117–124

Bald, E., Saigo, K., Mukaiyama, T. (1975) Facile synthesis of carboxamides by using 1-methyl-2-halopyridinium iodides as coupling reagents. *Chem. Lett.* 1163–1166

Gillberg, C. (1988) The neurobiology of infantile autism. *J. Child Psychol. Psychiatry* **29**: 257–266

Inhoffen, H. H., Nordsiek, K. H., Schäfer, H. (1963) Structure determination of an indole dye from *N*-[3-(3-indolyl)acryloyl]glycine. *Justus Liebigs Ann. Chem.* **668**: 104–121

Kimmig, J., Sticherlung, W., Tschesche, R., Urbach, H.-G. (1958) Isolation of N-[$\beta$(3-indolyl)acryloyl]glycine from urine and its synthesis. *Hoppe-Seyler's Z. Physiol. Chem.* **311**: 234–238

Knivsberg, A.-M., Wiig, K., Lind, G., Nødland, M., Reichelt, K.-L. (1990) Dietary intervention in autistic syndromes. *Brain Dysfunction* **3**: 315–327

Mandell, A. J., Rubin, R. T. (1965) Indolylacryloylglycine excretion in man. *Life Sci.* **4**: 1657–1664

Marklová, E. (1999) Where does indolylacrylic acid come from? *Amino Acids* **17**: 401–413

Marklová, E., Makovicková, H., Krákorová, I. (2000) Screening for defects in tryptophan metabolism. *J. Chromatogr. A* **870**: 289–293

Mills, M. J., Savery, D., Shattock, P. E. G. (1998) Rapid analysis of low levels of indolyl-3-acryloylglycine in human urine by high-performance liquid chromatography. *J. Chromatogr. B* **712**: 51–58

Milne, M. D., Crawford, M. A., Girao, C. B., Loughridge, L. (1960) The metabolic disorder in Hartnup disease. *Q. J. Med.* **29**: 407–421

Panksepp, J. (1979) A neurochemical theory of autism. *Trends Neurosci.* **2**: 174–177

Shattock, P. E. G., Kennedy, A., Rowell, F. J., Berney, T. (1990) Role of neuropeptides in autism and their relationships with classical neurotransmitters. *Brain Dysfunction* **3**: 328–345

Smith, H. G., Smith, W. R. D., Jepson, J. B. (1968) Interconversion of indolic acids by bacteria and rat tissue. Possible relevance to Hartnup disease. *Clin. Sci.* **34**: 333–342

Strohmayer, G. (1964) Nachweis von indolylacrylsäure bei der Phenylketonurie. *Arch. Klin. Exp. Dermatol.* **219**: 743–748