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High-performance liquid chromatographic profiling of indolylacryloylglycine and its possible precursors in body fluids

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Abstract

Indolylacryloylglycine (IAcrGly) is one of the physiological components of urine, although its source and its role in the human organism have not yet been unambiguously established. Changes in the IAcrGly excretion level have been found under some physiological (age dependence, seasonal variations) and pathological (photodermatoses, muscle dystrophy, liver cirrhosis) conditions. The proposed method for IAcrGly, indolylacrylic acid and its possible precursors, namely indolyllactic and indolylpropionic acids, involves deproteinization and extraction of urine on a Sep Pak C_{18} cartridge. HPLC analysis was carried out using a DataApex liquid chromatograph, equipped with an LCD 2082 UV detector, signals being acquired with a CSW workstation. The chromatographic column was Spherisorb ODS, 5 μm (125 × 4 mm I.D.), the mobile phase for isocratic elution was ethanol–1% acetic acid (27:73) and the flow-rate was 0.7 ml/min. The lower response limit is about 1 μmol/l for all metabolites at 280 nm.

Keywords: Indolylacryloylglycine; Tryptophan; Indolylacrylic acid; Indolyllactic acid; Indolylpropionic acid

1. Introduction

Tryptophan belongs among the amino acids with the highest number of metabolites and alternative metabolic degradative routes. In addition to the main pathways of catabolism, there exist minor and less thoroughy investigated routes; one of them leads to indolylacrylic acid (IAcrA).

In plants, IAcrA is included among growth hormones [1], whereas its role in animals is still obscure, as is the way in which and the site where it is formed in the organism. A two-stage production is likely: intestinal microorganisms catabolize tryptophan to indole derivatives, which are then absorbed and converted in the liver or kidney into IAcrA and its glycine conjugate, indolylacryloylglycine (IAcrGly) [2]. Our finding of IAcrGly in the urine of proven germ-free piglets points to the possibility that tryptophan can be converted into IAcrA without the intervention of intestinal microorganisms [3].

IAcrGly is a regular constituent of human urine [4] and it has been found in monkey, pig, rat and mouse [2], but the biological role of this substance in animals is unknown. Changes in its excretion have been observed under some physiological and pathological conditions [3,4], but, without any detailed knowledge of this metabolic pathway from tryptophan, those findings are difficult to explain.

The method for the determination of IAcrGly

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and IAcrA used until now is based on their unusual behaviour on a Sephadex G-10 column and involves, in addition, urine extraction, two-dimensional cellulose TLC and UV spectrophotometry [5]. Further study of IAcrGly and its potential precursors, which might be present in biological fluids in exceedingly small amounts, requires the introduction of a new analytical method.

There have been many reports of the isolation, HPLC separation and measurement of multiple tryptophan metabolites [6–9]; however, these investigations have generally not included indolylacryloylglycine. In this paper, we describe a reversed-phase HPLC method that allows the isocratic separation of IAcrGly, IAcrA and some of its possible precursors, namely indolylpropionic (IProA) and indolyllactic (ILA) acids, in urine and plasma.

2. Experimental

2.1. Reagents and materials

Authentic standards included indolyl-3-acryloylglycine (synthesized in the laboratory), indolyl-3-acrylic acid and indolyl-3-propionic acid (Fluka, Buchs, Switzerland), indolyl-3-acetic acid (IAA) (ICN, Costa Mesa, CA, USA), indolyl-3-lactic acid and indolyl-3-pyruvic acid (IPyrA) (Aldrich Europe, Beerse, Belgium), indolyl-3-butyric acid (IBA) (Loba Chemie, Vienna, Austria) and tryptophan (Trp) (Lachema, Brno, Czech Republic). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany) and ethanol, acetic acid and all other chemicals were of analytical-reagent grade from Lachema. Doubly-distilled water was used.

The mobile phase was prepared by mixing ethanol and 1% acetic acid in the proportions 27:73 (v/v) (A) or 40:60 (v/v) (B). The mixture was degassed with helium at the beginning of each day and passed through a 0.2-μm filter (Tessek, Prague, Czech Republic) before use.

Stock standard solutions (1 mg/ml) of indole derivatives were prepared by dissolving the substances either in the water (IAcrGly and Trp) or in mobile phase B, stored at −20°C and diluted 40–125-fold to give working standard solutions before use. The amounts of standards per injection volume of 20 μl were 160 ng of IAcrGly and Trp, 200 ng of IAA, 320 ng of IProA, 350 ng of IAcrA, 400 ng of ILA and IBA and 500 ng of IPyrA.

Phosphate buffer, 0.2 mol/l (pH 6), was prepared by dissolving 2.76 g of $NaH_2PO_4 \cdot H_2O$ in water, adjusting the pH with 0.1 mol/l NaOH and diluting to 100 ml.

Urine samples were collected for 24 h without preservatives and stored frozen at −20°C until analysis. Urine calibration standards were prepared by adding standard solutions to undiluted urine to provide the final concentration of 1–100 μg/ml.

Human heparinized plasma was obtained by centrifuging blood at 1500 *g* and 25°C for 10 min.

Each urine or plasma sample was supplemented with 20 μg of IBA as internal standard.

2.2. Deproteinization

Urine (1 ml) was deporteinized by adding 1 ml of aqueous zinc sulphate solution (100 g/l) and 1 ml of aqueous sodium hydroxide solution (1 mol/l), vortex mixed and mixed with 1 ml of phosphate buffer (pH 6). The whole supernatant was used for extraction. Proteins in heparinized plasma were precipitated with an equal volume of trichloroacetic acid in water (5 g per 100 ml), the supernatant was adjusted to pH 3–4 with sodium hydroxide (2 mol/l) and 100-μl aliquots were used for the next process.

2.3. Instrumentation

All measurements were performed with an HPLC apparatus (DataApex, Prague, Czech Republic), consisting a Model Micropump LCP 3001, Rheodyne Model 7125 injector and LCD 2082 UV detector; signals were acquired with a CSW (DataApex) workstation.

The chromatographic column was 125 × 4 mm I.D. Spherisorb ODS-2 (5 μm particle size), connected with a 4 × 4 mm I.D. LiChrospher 100

RP-18 (5 μm) guard column (both purchased from Hewlett-Packard, Amsterdam, Netherlands).

2.4. Chromatographic conditions

For the isocratic separation of sample components, the flow-rate of mobile phase A was kept at 0.7 ml/min and the effluent was monitored at 280 or 323 nm. All chromatographic runs were performed at 22–24°C.

2.5. Solid-phase extraction

A modified method of Tonelli et al. [10] was used. Disposable Sep Pak C_{18} extraction cartridges, 100 mg (Waters, Milford, MA, USA), were activated by washing them with 5 ml of methanol followed by 5 ml of phosphate buffer (pH 6). A 1-ml volume of standard solution, deproteinized urine or plasma, spiked with 20 μg of internal standard (I.S.) was loaded on the activated cartridge and the sample passed through a sorbent. The cartridge was then washed with 1 ml of water and indole derivatives were eluted with 2 ml of methanol. The eluate was evaporated in vacuo and the residue dissolved in 250 μl of mobile phase A, passed through a 0.2-μm Anotop 10 filter (Merck) and a 20-μl aliquot was injected into the HPLC system. A slow, dropwise flow during the sample pretreatment was maintained in order to achieve the optimum recovery. All manipulations were carried out in the dark.

3. Results and discussion

3.1. Assignment of peaks

Peak assignments were made by comparing the retention times of the peaks with those of known compounds and by injecting known compounds together with the samples. In addition to the precursors of IAcrGly, two other indolic compounds with similar chromatographic parameters, usually present in normal urine, were tested, namely Trp and IAA (Fig. 1).

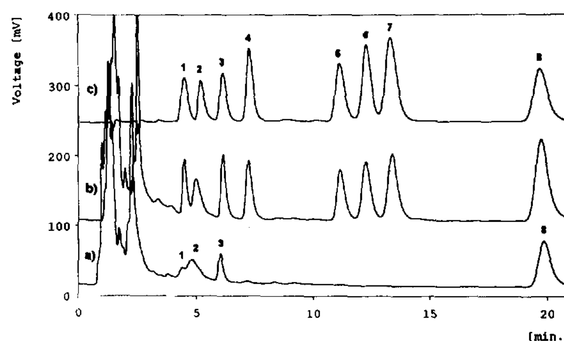

Fig. 1. Chromatograms of (a) urine from a 3-month-old infant, (b) urine spiked with standards and (c) standard mixture. Peaks: 1 = indolylacryloylglycine; 2 = tryptophan; 3 = indolyllactic acid; 4 = indolylacetic acid; 5 = indolylpropionic acid; 6 = indolylpyruvic acid; 7 = indolylacrylic acid; 8 = indolylbutric acid. Detection wavelength, 280 nm. HPLC conditions are described under Experimental.

For quantitative purposes, plots of peak heights at 280 nm versus amount of sample injected were performed for IAcrGly, IAcrA, ILA and IProA. The results were calculated using the internal standard method. The calibration graphs were found to be linear over the range of amounts tested, i.e., up to 100 μg/ml ($r = 0.996$ for IAcrGly).

The detection of IAcrA and IAcrGly is more specific and even more sensitive at 323 nm, where most other indolic compounds does not absorb.

3.2. Stability

Whereas stock standard solutions may be used for at least 3 months, the working standard solutions are recommended to be prepared fresh before injection, because most of the compounds are easily degraded in organic solvents [8]. The greatest instability was shown by standard solutions of IPyrA.

3.3. Deproteinization

We tried to simplify the procedure by omitting the protein removal step, but the results were better when deproteinized urine was used, as recommended by Tonelli et al. [10].

3.4. Reproducibility of the separation

To demonstrate the reproducibility of the standard separation system, repeated chromatographic analyses were performed on the same urine sample, supplemented with the standard substances (amounts in a 20-μl injection volume are described under Experimental). The retention times were determined and were found to have a relative standard deviation of 0.8–2.0%. Hence retention time is useful for the qualitative analysis of a substance. The peak heights of the standards were determined and found to have a relative standard deviation of 1.6–3.1%. This peak height could be useful for quantitative analysis. The analytical recovery ranged from 97.1 to 99.3%.

Allowing a signal-to-noise ratio of 3, the detection limits were calculated and the results are reported in Table 1. The values ranged from 6.0 to 18.5 ng per 20 μl injected at a wavelength of the UV detector of 280 nm and 2.9 ng for IAcrGly and 4.0 ng for IAcrA at 323 nm. Unfortunately, the method does not seem to be fully satisfactory for IPyrA analysis, mainly because of its great instability under the conditions used.

We found that a Sep Pak C_{18} cartridge could be re-used many times (up to twelve) without any loss of performance, which is in good agreement with the literature [11].

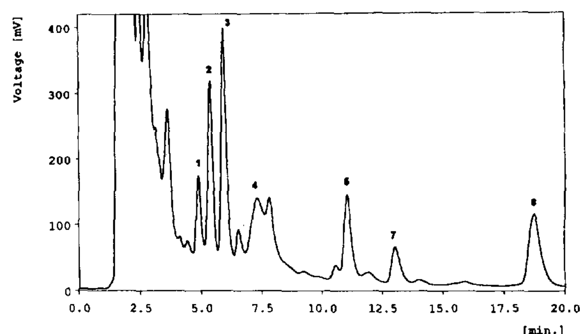

Fig. 2. Typical chromatogram of urine from a healthy adult. For details, see Fig. 1.

We tested the adaptability of these techniques for the analysis of urine samples from healthy adults and from infants suffering from various diseases. A typical chromatogram of urine from a healthy adult is shown in Fig. 2.

Preliminary results testify to the possibility of adapting the chromatographic system for monitoring IAcrA, IProA and ILA in plasma (Fig. 3).

In conclusion, the present method, although still open to further improvement, is reliable for the simultaneous determination of IAcrGly, IAcrA and its two possible precursors, using a

Table 1
Elution times, reproducibility, recoveries and detection limits

Compound	Elution time (min)	R.S.D. (%)	Peak height (mV)	R.S.D. (%)	Recovery[a] (%)	R.S.D. (%)	Detection limit[b]	
							At 280 nm	At 323 nm
IAcrGly	4.42	0.8	74.2	2.0	98.7	1.9	6.0	2.9
Trp	4.98	1.7	67.1	3.1	–	–	–	–
IAA	6.02	1.3	75.1	1.8	–	–	–	–
ILA	7.13	1.9	106.3	2.8	97.1	4.0	18.5	–
IProA	11.09	1.0	81.1	1.6	99.2	3.2	16.0	–
IAcrA	13.33	0.9	117.2	1.6	98.0	1.1	7.5	4.0
IBA (I.S.)	19.76	2.0	74.5	2.2	99.3	1.1	18.0	–

Each value is an average of eight independent analyses (day-to-day).
[a] Recoveries were measured by repeated injection of urine samples spiked or not with 20 μl of working standard solutions (for details, see Experimental).
[b] Injected amount (ng in 20 μl) of standards giving a signal-to-noise ratio of 3.

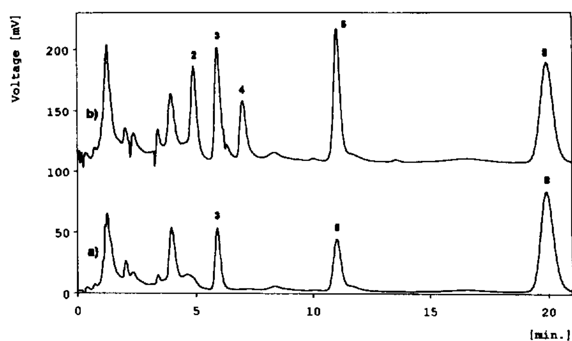

Fig. 3. Chromatograms of (a) plasma from a 3-month-old infant and (b) plasma spiked with some of the standards. For details, see Fig. 1.

relatively simple and rapid procedure, which is adaptable for routine analysis of urine and plasma.

Acknowledgements

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