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Short communication

Screening for defects in tryptophan metabolism

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Abstract

We introduced a two-step procedure for the detection of defects in metabolism of tryptophan: (1) HPTLC (described previously) is suitable when starting the investigation, (2) two HPLC methods with isocratic elution and spectrophotometric detection are used at the next step, when pathological findings are to be confirmed and the individual metabolites quantified. The first method enables the assessment of tryptophan, 5-hydroxyindolylacetic acid, indolylacetic acid, indolylacryloylglycine, indolylacrylic acid and its possible precursors, namely indolyllactic and indolylpropionic acids. The second procedure is intended for the monitoring of anthranilic, 3-hydroxyanthranilic, kynurenic and xanthurenic acids, kynurenine, 3-hydroxykynurenine and indoxyl-sulfate. The same pre-treated sample is used for all methods.

Keywords: Tryptophan

1. Introduction

Tryptophan (Trp) in humans is catabolized by several pathways, the main one leading to kynurenine metabolites with an important side-product, nicotinamide; other ways lead to the formation of various indolic compounds including 5-hydroxy derivatives, namely serotonin.

A number of diseases is connected with abnormalities in the metabolism of Trp, but the relationship of cause and effect is usually unclear. Concomitant clinical symptoms include the skin, intestinal, hematological, neurological and psychiatric defects [1,2].

Employment of various chromatographic-group tests in the screening of metabolic disorders has, in general, proved to be very effective [3]. Many excellent reports have been published on the separation and measurement of multiple Trp metabolites, employing various analytical methods (for review, see [4]), reversed-phase liquid chromatography being a method of choice due to its rapidity and efficiency. However, a lot of reports assume the availability of gradient elution, highly sensitive fluorimetric or electrochemical detectors [5–8], changes of flow-rate, column and detector type or the wavelength during the analysis [9–12], which facilities are not always available in clinical laboratories.

We have tested and modified (sample extraction, mobile phase, type of detector) some of those procedures, applicable in our laboratory [12–15], introducing an algorithm for routine analysis.

The best results have been achieved using a Sep-Pak pre-treatment, combined (1) with HPTLC on cellulose (the method was described in [16]) for the first step of screening, when a rather large number of patients is to be tested. (2) Two HPLC methods with

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isocratic elution and spectrophotometric detection are used as a complement at the second step, in particular whenever pathological findings should be confirmed and the metabolites quantified. The same Sep-Pak pre-treated sample is used for all procedures.

We have previously described [17] and later modified the first HPLC method, suitable for the assessment of tryptophan, 5-hydroxyindolylacetic acid (5HIAA), indolylacetic acid (IAA), indolacryloylglycine (IAcrGly), indolylacrylic acid (IAcrA) and its possible precursors, namely indolyllactic and indolylpropionic acids (ILA, IProA) (group A).

The second procedure is intended to monitor anthranilic (AA), 3-hydroxyanthranilic (3HAA), nicotinic (NA), kynurenic (KA) and xanthurenic (XA) acids, kynurenine (Kyn), 3-hydroxykynurenine (3HKyn) and indoxyl-sulfate (IS) (group B).

The methods, routinely used for urine analysis, are applicable for other matrices, such as serum, plasma or cerebrospinal fluid.

2. Experimental

2.1. Materials

All standards were of analytical reagent grade, purchased from Sigma (St. Louis, MO, USA) Merck (Darmstadt, Germany) and Aldrich (Milwaukee, WI, USA). Sep-Pak Plus C_{18} cartridges were supplied by Waters (Milford, MA, USA), solvents (HPLC grade) were obtained from Merck.

Concentrated stock solutions of standards (0.1 mg ml^{-1}) group A were prepared in deionized water or 25% ethanol, standards group B and the mixture of multiple standards were prepared in (hot) acetate–citrate buffer, pH 4.5, both stored at −20°C, daily diluted (3–20×) with the buffer for working solutions. The amounts of standards (in μg per injection volume of 20 μl) were: 0.08 of 5HIAA, 0.2 of IAcrGly and IAA, 0.3 of IAcrA, 0.4 of ILA, Trp and IProA (group A) and 0.17 of NA, 0.2 of 5HIAA, 0.2 of Kyn, 0.4 of 3HKyn and 3HAA, 0.5 of Trp, XA, KA and AA, 0.8 of IS (group B).

(Physiologically) protein-free urine was checked for creatinine concentration and the volume containing 2 μmol creatinine was diluted with the 0.04 *M* acetate–citrate buffer, pH 4.5, making a total volume of 3 ml. Serum or cerebrospinal fluid (also urine, if proteinuria was found) were deproteinized with 5% sulfosalicylic acid (1:1, v/v), vortex-mixed and centrifuged at 1800 *g* for 15 min. The supernatant was withdrawn, alkalized to pH 5 (2 *M* sodium hydroxide) and made up to 1 ml using the buffer, pH 4.5. All samples were then applied to the Sep-Pak cartridge.

2.2. Instruments

Solid-phase extraction (SPE) vacuum manifold for Sep-Pak sample pre-treatment was purchased from Baker (Phillipsburg, NJ, USA). Thermovap unit for sample evaporation supplied Ecom (Prague, Czech Republic). Analyses were performed using Ecom (Prague, Czech Republic) liquid chromatograph equipped with LCP 3001 micropump, LCD 2084 processor–UV detector, GP3 three-channel solvent delivery system and type 234 Gilsone autosampler (Middleton, USA) with Rheodyne injector. DataApex (Prague, Czech Republic) workstation was used to acquire the elution data. The analytical column was 125×4 mm I.D., 5 μm particle diameter, containing HP-Spherisorb RP-18 ODS 2 stationary phase in a LiChroCart cartridge (Hewlett-Packard, The Netherlands).

2.3. Solid-phase extraction

The Sep-Pak cartridges were wetted with 5 ml methanol and conditioned with 2 ml 0.01 *M* sodium dodecylsulfate (SDS, pH 2.1). The samples (pH 4.5) were then applied and drawn through the cartridges under vacuum with a flow-rate of approximately 1 ml min^{-1}. The impurities were gradually washed out from the sorbent with l ml 0.01 *M* SDS and 1 ml methanol–0.01 *M* SDS (5:95), the analytes were eluted with 4 ml of 1 *M* ammonia–methanol (80:20). The eluates were evaporated under nitrogen at 25°C, redissolved in 500 μl methanol and filtered.

The pre-treated sample was then used for HPTLC and for both HPLC procedures; an aliquot of the sample was diluted with the respective mobile phase (1:1, v/v), the injection volume was 20 μl.

2.4. Chromatographic conditions

For the isocratic separation of metabolites a flow-rate 0.7 ml min^{-1} was used. The mobile phases were filtered and degassed by sonication. Using a central filling of 100-μl loop, 20 μl of sample was injected in duplicate and all chromatographic runs were performed at 22–24°C.

Procedure I: The mobile phase was prepared by mixing 1% acetic acid–ethanol (75:25), the effluent was monitored at 280 and 323 (IAcrGly and IAcrA) nm.

Procedure II: The mobile phase consisted of 0.04 *M* acetate–citrate buffer, pH 4.5 with 2.5% (v/v) acetonitrile, the detector was set at 254 (280, 325) nm.

3. Results and discussion

Typical chromatograms of a synthetic mixture of reference compounds are shown in Figs. 1 and 2. High IAcrGly excretion in a patient with Duchenne type muscle dystrophy and the benign xanthinuria in a child are presented as examples of pathological findings.

Assignment of peak identities was made on the basis of retention times, by spiking of unknown sample with standards, by checking the spectral characteristics and using different wavelength of detector. Since XA occasionally overlaps with Trp at 254 nm, checking XA at 325 nm might facilitate the identification. Comparison with the results received on HPTLC, viewed under UV light and sprayed with Ehrlich reagent might help when doubt arises [13,16].

Peak areas versus amount of injected standards were checked for quantification, using an external standard technique. The calibration graphs were found to be linear up to 100 μg/ml.

Procedure I. Detection limits were 6.0–18.5 ng per 20 μl injected, *S*/*N* ratio of 3, with the detector set to 280 nm, 2.9–4.0 ng per 20 μl for IAcrGly and IAcrA, checked at 323 nm. Recovery of metabolites (urine spiked with the standard substances at concentration 0.1 mmol l^{-1}, $n=8$) varied between 88.2 and 99.3%. Reproducibility was tested on the same urine sample, supplemented with the standard sub-

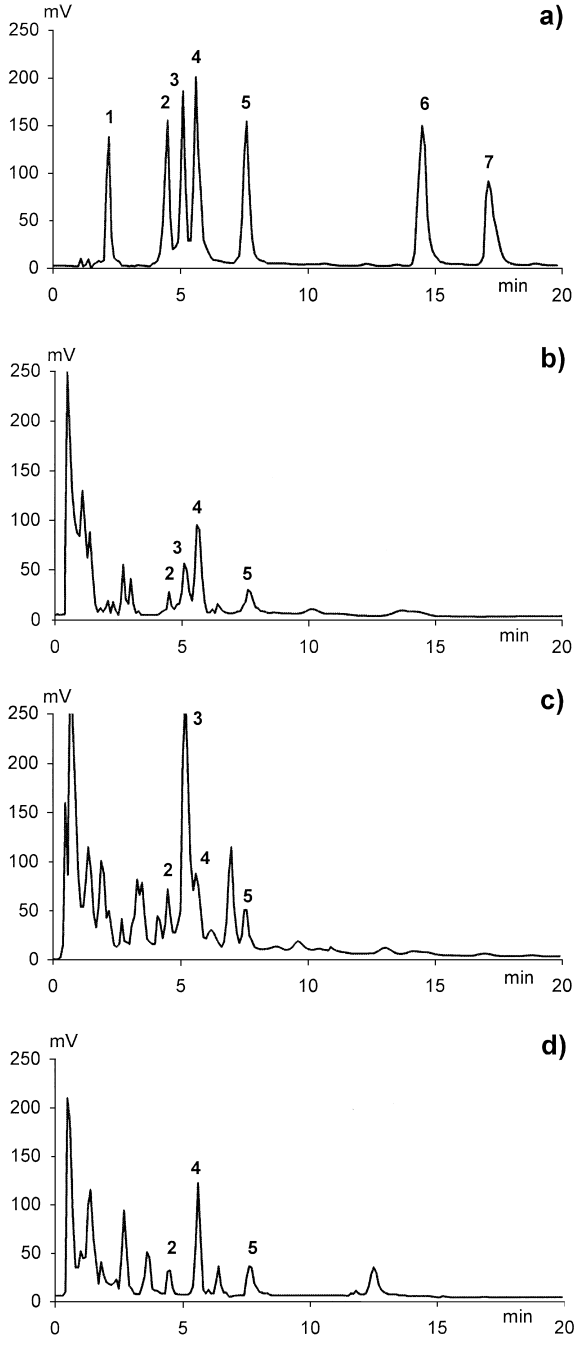

Fig. 1. Procedure I. HPLC chromatograms of (a) mixture of standards (group A), (b) control urine (child), (c) urine of a boy with the Duchenne type muscle dystrophy, (d) control plasma (child); 1, 5-hydroxyindolylacetic acid; 2, indolyllactic acid; 3, indolylacryloylglycine; 4, tryptophan; 5, indolylacetic acid; 6, indolylpropionic acid; 7, indolylacrylic acid. For details see Experimental.

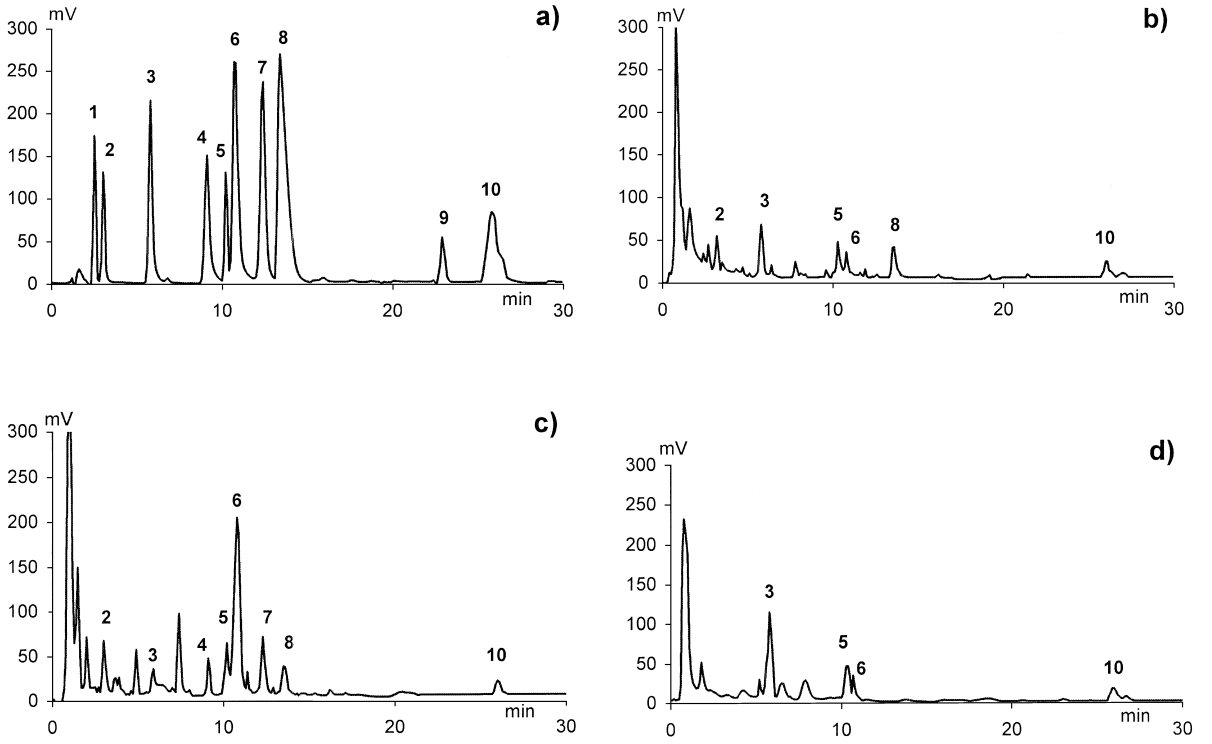

Fig. 2. Procedure II. HPLC chromatograms, detector set to 254 nm. (a) Mixture of standards (group B), (b) control urine (child), (c) urine of a child with benign xanthurenic aciduria, (d) control plasma (child); 1, nicotinic acid; 2, 3-hydroxykynurenine; 3, kynurenine; 4, 3-hydroxyanthranilic acid; 5, tryptophan; 6, xanthurenic acid; 7, kynurenic acid; 8, 3-indoxyl sulfate; 9, 5-hydroxyindolylacetic acid; 10, anthranilic acid. For details see Experimental.

stances (six parallel analyses, day-to-day). Results for the retention times were found to have a relative standard deviation (RSD, %) of 0.8–1.2 and for the peak heights the RSD was in the range of 1.6–2.2.

Procedure II. Detection limits were 5.0–22.0 ng per 20 μl injected at a wavelength of 254 nm. Recovery of metabolites (0.1 mmol l^{-1}, $n=8$) was between 80.0 and 98.5%, reproducibilities of the retention times and the peak heights were 0.9–1.1 and 1.3–3.2, respectively (expressed as an RSD value, tested under the conditions described above). Trp and 5HIAA were also detected.

Trp metabolites are difficult to analyse because of their lability and low levels in biological fluids, thus prompt processing, immediate deproteinization, light protection and a low temperature were the conditions essential for obtaining good results. The manifold working under vacuum presents quick way of extraction, recovery of all metabolites being high enough (it varied between 82 and 99%).

The standard stock solutions were stable up to 3 months at −20°C, the working solutions were prepared daily and the mobile phase for the procedure II was mixed freshly after each 12 h of the run to achieve good retention time reproducibility.

We tried to use the native samples for HPLC, but have not been successful in getting good results, as the peaks with lower retention times overlapped with impurities (a large initial peak), which was expressed in some of the samples, whatever material was used.

It is important to follow the dietetic status of patients to overcome the possible nutritional deficiency and thus insufficient concentration of metabolites precursor in the diet, related to the sensitivity of the method, otherwise loading with Trp (100 mg per kg body mass) is recommended.

The procedure is applicable for the determination of the Trp metabolites in human urine, plasma or serum and cerebrospinal fluid. The method is selective and reliable for use in screening and diagnostic applications.

Acknowledgements

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