The hyperserotonemia of autism spectrum disorders

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Chapter 4

Automated On-Line Solid-Phase Extraction Coupled with HPLC for Measurement of 5-Hydroxyindole-3-acetic Acid in Urine

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Abstract

Background: Quantification of 5-hydroxyindole-3-acetic acid (5-HIAA) in urine is useful in diagnosing and monitoring of patients with carcinoid tumors and in the study of serotonin (5-hydroxytryptamine) metabolism in various disorders. We describe an automated method that incorporates on-line solid-phase extraction (SPE) and HPLC to measure urinary 5-HIAA. Methods: Automated prepurification of urine was accomplished with HySphere-resin GP SPE cartridges containing strong hydrophobic polystyrene resin. The analyte (5-HIAA) and internal standard [5-hydroxyindole-3-carboxylic acid (5-HICA)] were eluted from the SPE cartridge, separated by reversed-phase HPLC, and detected fluorometrically with a total cycle time of 20 min. Urinary excretion of 5-HIAA was measured in a group of patients with known and suspected carcinoid tumors (n = 63) and in 20 patients with autism. Results: The internal standard (5-HICA) and 5-HIAA were recovered in high yields (87.2%–114%). Within- and between-series CVs for the measurement of 5-HIAA in urine ranged from 1.2% to 3.9% and 3.2% to 7.6%, respectively. For urine samples from patients with known or suspected carcinoid tumors, results obtained by the automated method were highly correlated (r = 0.988) with those from an established manual extraction method. For samples from autistic patients, urinary excretion of 5-HIAA was similar to that reported for healthy individuals. Conclusion: This SPE-HPLC method demonstrated lower imprecision and time per analysis than the manual solvent extraction method.

Introduction

The neurotransmitter/neurohormone serotonin [5-hydroxytryptamine, (5-HT)] is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut and in serotonergic neurons in the central nervous system (Tyce, 1985; Young and Teff, 1989). Peripheral serotonin is metabolized mainly in the lung and the liver through the action of monoamine oxidase-A. 5-Hydroxyindole-3-acetic acid (5-HIAA) is the predominant end-product of serotonin metabolism and is subsequently excreted in urine (Grahame-Smith, 1988).

Serotonin is clearly involved in carcinoid syndrome and is hypothesized to play a role in schizophrenia, depression, migraine, and autism (Kema et al, 2000; Naughton et al., 2000; Anderson, 2002). The most pronounced deviations in serotonin metabolism are found in patients with carcinoid tumors, which can secrete large amounts of serotonin (Kema et al., 1999). Quantification of urinary 5-HIAA is important in the diagnosis and follow-up of such patients. Diagnosis can be established by measuring urinary 5-HIAA or platelet serotonin. When serotonin production of the tumor exceeds platelet storage capacity, however, changes in production will not be reflected in platelet serotonin concentrations, whereas changes in the amount of 5-HIAA excreted in urine will still occur (Kema et al., 1992). Increased platelet serotonin has also been observed in autistic patients, with typical reported group mean increases of 25%–50% (Anderson, 2002). For the study of peripheral serotonin metabolism in autism, measurement of 5-HIAA in urine is useful to evaluate a possible role of altered production or catabolism in observed changes in platelet serotonin (Anderson, 2002).

Since the early 1950s, many analytical methods have been described to measure 5-HIAA in urine (Deacon, 1994). The first methods used colorimetric methodology, paper and thinlayer chromatography, or gas chromatography. Later, various HPLC analyses were reported that used colorimetric, fluorometric, or electrochemical detection (Deacon, 1994). Recently, liquid chromatographic–tandem mass spectrometric methods have been reported for urine and whole blood (Kroll et al., 2002; Danaceau et al. 2003).

We developed an automated method with on-line solid-phase extraction (SPE) and HPLC with fluorometric detection to measure urinary 5-HIAA and applied this method to the determination of urinary 5-HIAA excretion rates in individuals with known or suspected carcinoid tumors and in individuals with autism.

Materials and Methods

Reagents and stock solutions

HPLC-grade acetonitrile and methanol were obtained from Rathburn; 5-HIAA and disodium EDTA were from Sigma; tetrabutylammonium bromide was from Romil; dipotassium EDTA was from Brunschwig; 5-hydroxyindole-3-carboxylic acid (5-HICA) was from Janssen Chimica; and acetic acid, sodium metabisulfite ($Na_2S_2O_5$), ascorbic acid, phosphoric acid, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were from Merck. All reagents were of at least analytical reagent grade. Reagent-grade water, obtained from a Barnstead system, was used throughout.

Stock solutions containing 0.34 mmol/L 5-HICA or 2.1 mmol/L 5-HIAA were prepared in water containing 20 mL/L glacial acetic acid. The 5-HICA stock solution was stored protected from light at -20 °C for a maximum of 1 year. The 5-HIAA stock solution was prepared on the day of analysis. Saturated stock solutions containing ascorbic acid (300 g/L), dipotassium EDTA (750 g/L), and $Na_2S_2O_5$ (750 g/L) were prepared in water and stored at room temperature for 3 weeks.

Samples and participants

Patient urine samples were collected into brown polypropylene bottles (Sarstedt) containing ~250 mg each of $Na_2S_2O_5$ and disodium EDTA. Urine samples were acidified to pH 4 with acetic acid and stored at -20 °C. Before analysis, aliquots of thawed urine samples (50 µL) were mixed with 300 µL of antioxidant solution (100 µL of ascorbic acid stock solution, 100 µL of dipotassium EDTA stock solution, and 100 µL of $Na_2S_2O_5$ stock solution) and 100 µL of internal standard stock solution (0.34 mmol/L 5-HICA). The mixture was then diluted with 1.2 mL of water, and 20 µL of each sample was injected into the SPE-HPLC system as described below. This injection volume was equivalent to 0.61 µL of urine.

For method-comparison studies, we used spot urine samples from 63 patients with suspected or known carcinoid tumors and 24-hr urine specimens from 20 individuals with autism (Mulder et al., 2004).

Analysis and quantification

Instrumentation. The Prospekt-2 (Spark Holland) on-line SPE unit consisted of 3 modules: an SPE controller unit (automated cartridge exchanger), a solvent delivery

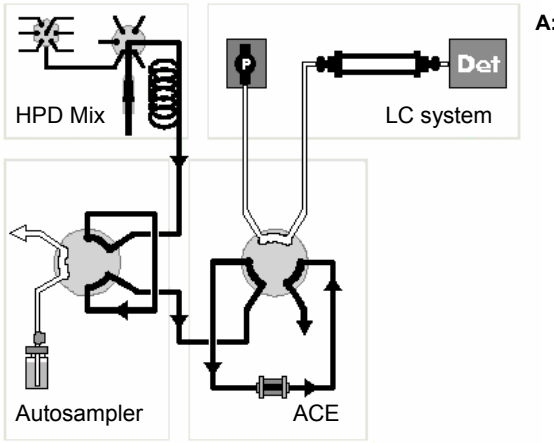
unit (high-pressure dispenser), and an autosampler (Triathlon). A SparkLink software package (Ver. 2.10) was required for control of the Prospekt-2 modules. The main task of the automated cartridge exchangers was exchange of disposable SPE cartridges. The high-pressure dispenser had a single-syringe configuration and delivered solvents for conditioning, sample application, and clean up of SPE cartridges. HySphere-resin GP cartridges (10 x 2 mm; Spark Holland) were used for cleaning up samples and concentrating analytes. Samples were injected with a Triathlon autosampler. The HPLC pump used was a Gynkotek Series P580A binary high-pressure gradient pump (Softron); detection was with a Waters 474 spectrofluorometer (excitation wavelength, 280 nm; emission wavelength, 360 nm). The temperature of the column was regulated with a Mistral column oven (Spark Holland).

For analytical HPLC, a 100 x 4.6 (i.d.) mm Brownlee Spheri-5 RP-18 column filled with 5-µm spherical particles (Inacom) was used. The analytical column was preceded by a 15 x 3.2 (i.d.) mm Aquapore RP18 ODS guard column filled with 7-µm spherical particles (Inacom). Detector output was integrated by ChromPerfect (Ver. 3.51) integration software (Justice Innovations).

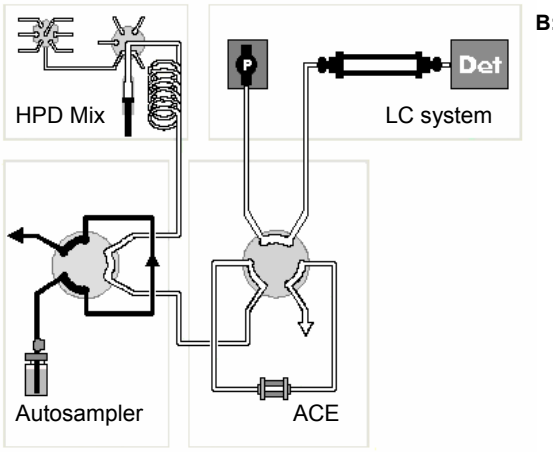
Eluents. The isocratic system consisted of eluent B (11 mmol/L dipotassium hydrogen phosphate and 3 mmol/L tetrabutylammonium bromide in water adjusted to pH 6.0 with phosphoric acid, added to 80 mL of acetonitrile to a total eluent volume of 1 L). Eluent A (22 mmol/L potassium dihydrogen phosphate in water adjusted to pH 2.0 with phosphoric acid, added to 10 mL of acetonitrile to a total eluent volume of 1 L) was delivered to the high-pressure dispenser.

Before use, eluent B was filtered through a 0.45 µm membrane filter (Schleicher and Schuell) and was degassed before entering the HPLC pump. The flow rate of eluent B was 1.0 mL/min, and chromatography was performed at 20 °C.

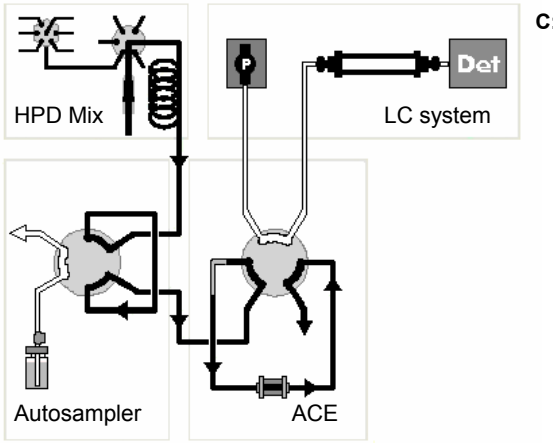
On-line SPE. On-line SPE was performed according to the schedule specified in Table 4.1 and as depicted schematically in Figure 4.1. The system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge was loaded, purged for clean up, and eluted to the analytical column. During the loading routine, the SPE cartridge was conditioned sequentially with methanol, eluent A, 5 g/L dipotassium EDTA in water, and eluent A, all provided by the highpressure dispenser at flow rates of 2000 µL/min. The mixing coil between the



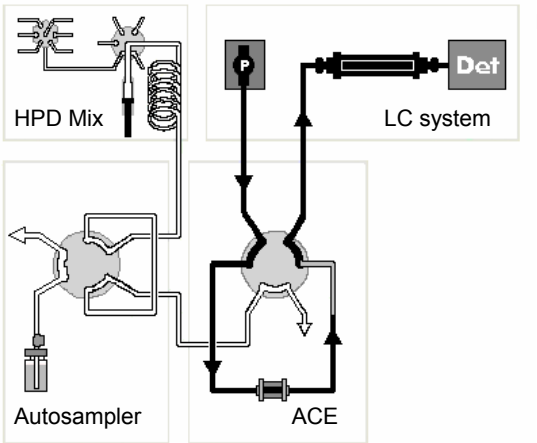
A: Solvation and equilibration



B: Loopfill Autosampler



C: Sample to cartridge



D: Elution sample to column

Figure 4.1: Schematic representation of the on-line SPE system coupled to HPLC with fluorescence detection. (A), solvation and equilibration step, showing the system during conditioning of the cartridge. (B), filling of the autosampler loop, showing the sample as it is processed by the autosampler. (C), flow of sample to cartridge, showing the system as the sample is on its way to the cartridge, where it will be washed. (D), elution of sample from the cartridge to the column, showing the system during the elution of the cartridge. After this, the system can be programmed to change the cartridge before the next sample is introduced. For additional details, see Table 1 and the Materials and Methods. HPD, high-pressure dispenser; LC, liquid chromatography; P, pump; Det, detector; ACE, automated cartridge exchanger.

Table 4.1: Summary of the sequence of events used for on-line SPE, as described in Materials and Methods.

Step	Action	Details		Comment
01	New cartridge	Left clamp		Put cartridge in clamp
02	Solvation	Methanol (2000 µL at 2000 µL/min)	SSM 1A	Activate cartridge with methanol
03	Equilibration	Eluent A (2000 µL at 2000 µL/min)	SSM 1B	Condition cartridge with eluent A
04	Equilibration	5 g/L dipotassium EDTA (2000 µL at 2000 µL/min)	SSM 1D	Condition cartridge with dipotassium EDTA
05	Equilibration	Eluent A (2000 µL at 2000 µL/min)	SSM 1B	Condition cartridge with eluent A
06	Start autosampler	Start loopfill		Fill loop autosampler with sample
07	Sample application	Eluent A (2000 µL at 2000 µL/min)	SSM 1B	Inject sample (load sample on cartridge using eluent A)
08	Wash cartridge	Eluent B (560 µL at 1000 µL/min)	SSM 1C	Wash cartridge with eluent B (removal ofinterferents)
09	Inputs	Input 1 high	Pump ready?	
10	Outputs	Auxiliaries 1 and 2	Start pump and start data	
11	Elution	1.5 min		Elution of sample from cartridge to analytical column
12	Move cartridge	Left to tray		Cartridge back to tray

high-pressure dispenser and the autosampler facilitated mixing of the eluents before they reached the cartridge.

After the mixed eluents reached the cartridge, the autosampler injected the sample, thereby loading the cartridge with the sample, using eluent A. The highpressure dispenser then continued to wash the loaded cartridge with eluent B, thus cleaning the cartridge; the analytes were then eluted from the cartridge to the analytical column with eluent B in 1.5 min. After elution from the cartridge, chromatographic separation on the analytical column occurred. During this separation, the next sample was loaded on the cartridge and washed. When the pump was ready, this sample was eluted to the analytical column. If necessary, cartridges could be changed automatically.

5-HIAA was quantified by calculation of the peak-area ratios in samples relative to those of the internal standard (5-HICA). Sample peak-area ratios were compared with the peak-area ratios obtained for the calibration solutions at 6 different concentrations, which were prepared by addition of known amounts of 0.21 mmol/L 5-HIAA calibrator solution (prepared fresh daily by diluting the stock solution 10-fold with water).

Urine creatinine was measured by a picric acid method on a Merck Mega Analyzer.

Analytical characteristics

In on-line SPE, detection limits depend on the extent of sample preconcentration on the SPE cartridge. For urine, we determined detection limits by injecting samples with decreasing concentrations of 5-HIAA. The detection limit was defined as the injected amount that produced a signal-to-noise ratio of 3. We estimated the percentage of carryover between sequential analyses performed on a single SPE cartridge by alternating injections of blanks and urine samples with high concentrations of 5-HIAA.

Quality control and validation of the automated urinary 5-hiaa method

Recoveries were estimated by the addition of 5-HIAA in 3 different concentrations (160, 350, and 520 µmol/L) to 1 urine specimen. Recoveries were measured in 6 replicates of these samples and calculated relative to the recovery of the internal standard; mean recoveries and ranges are reported. Within- and between-series precision was determined by use of 3 samples with 5-HIAA concentrations in the low, medium, and high ranges. Within-series precision was assessed in 6 replicates analyzed in a single series; between-series precision was assessed on 6 different days over a 1-month period. Experiments were performed using 1 SPE cartridge per series.

The identities of sample 5-HIAA peaks were verified by addition of calibrator (standard addition) and by observation of retention time changes of the sample peak under different chromatographic conditions. The automated urinary 5-HIAA method was also validated by comparison of its results with those obtained by our routinely used manual ether extraction method (Rosano et al., 1982).

Statistics

We compared methods by Deming regression analysis using the slope and intercept; we also calculated the correlation coefficient and the standard error of prediction ($S_{y|x}$). The Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) EP-6P protocol (Passey et al., 1986) was applied to test the linearity of the method.

Results

Chromatography

The chromatographic separation of 2 calibrators by automated on-line SPE and HPLC with fluorometric detection is shown in Figure 4.2A. Total cycle time was ~20 min (Table 4.1). Chromatograms of urine samples obtained from a healthy adult and from a patient with a carcinoid tumor are shown in panels B and C, respectively, of Figure 4.2 (note the different sensitivity scales). Complete separation of the analytes from interferents was achieved within 16 min. Comparison of the two chromatograms shows markedly increased 5-HIAA in the sample from the patient.

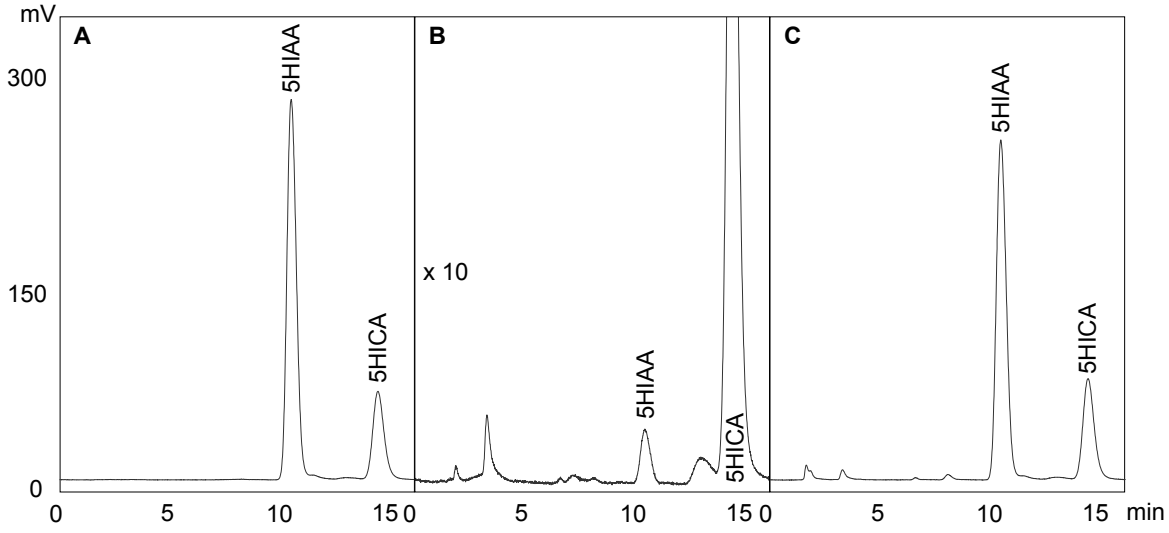


Figure 4.2: Chromatograms of urine from a healthy adult and a patient with carcinoid tumor, as obtained by on-line SPE coupled to HPLC with fluorescence detection. Excitation wavelength, 280 nm; emission wavelength, 360 nm. (A), separation of 5-HIAA and the internal standard 5-HICA (injected amounts, 0.34 nmol for both). (B), 5-HIAA in urine from a healthy adult (note the 10-fold increase in sensitivity). (C), 5-HIAA in urine obtained from a patient with carcinoid tumor. Chromatograms in B and C were obtained with 20 µL injections of diluted urine (equivalent to 0.61 µL of urine). Calculated 5-HIAA concentrations in the urine samples were 8.75 and 506 µmol/L for B and C, respectively.

Analytical characteristics

A signal-to-noise ratio of 3 was achieved at a 5-HIAA concentration of 0.8 µmol/L. Analysis of a split urine specimen with increasing amounts of added 5-HIAA calibrator demonstrated that the assay was linear to 5-HIAA concentrations of at least 2000 µmol/L. The percentage carryover between sequential analyses performed on a single SPE cartridge was <1%.

Quality control and validation of the automated urinary 5-hiaa method

The results of the recovery and precision experiments are presented in Table 4.2. The recovery data represent recoveries for 6 replicates of each of 3 samples

analyzed in 1 analysis time series with 1 SPE cartridge. Recoveries ranged from 87.2% to 114.0%. Within-series imprecision (as CVs) based on 6 measurements of 3 different urine samples were 3.9%, 1.6%, and 1.2% in samples with mean (SD) 5-HIAA concentrations of 2.3 (0.1), 25.0 (0.4), and 147.9 (1.7) µmol/L, respectively. The between-series imprecision (CVs) for analyses performed across 6 series were 7.6%, 3.7%, and 3.2% in samples with 5-HIAA concentrations of 2.5 (0.2), 26.3 (1.0), and 154.8 (3.2) µmol/L, respectively.

Table 4.2: Recovery and precision of the on-line SPE urinary 5-HIAA excretion method

Recovery[a]	
Added, µmol/L[b]	*% (range)*
160	94.0 (87.2-98.9)
350	99.9 (95.0-109.1)
540	102.1 (95.8-114.0)

Precision[c]			
Within-series		*Between-series*	
5-HIAA, µmol/L, (mean ± SD)	*CV, %*	*5-HIAA, µmol/L, (mean ± SD)*	*CV, %*
2.3 ± 0.1	3.9	2.5 ± 0.2	7.6
25.0 ± 0.4	1.6	26.3 ± 1.0	3.8
147.9 ± 1.7	1.2	154.8 ± 3.2	3.2

NOTE: a. Recovery data represent data from 6 measurements of each supplemented sample determined in single series using one SPE cartridge. b. The endogenous concentration of 5-HIAA in the urine sample used in the recovery experiments was (mean ± SD): 29.2 ± 1.2 µmol/L. c. Within-series precision data are derived from 6 measurements of three samples (low, mid and high concentration) made in a single series. Between-series data are from measurements of the same three samples made in 6 different series using one SPE cartridge per series.

As seen in Figure 4.3, 5-HIAA values determined in 63 clinical samples by the automated SPE method were highly correlated with results obtained by the manual ether extraction method (Rosano et al., 1982). Deming regression gave a slope of 0.9996 (range, 0.9811–1.0181) and a y-intercept of -3.50 µmol/L (95% confidence interval, -9.92 to 2.92 µmol/L). The Spearman correlation coefficient was 0.988 ($p < 0.0001$), and the SE of prediction ($S_{y|x}$) was 3.21 µmol/L. The lack of linear fit test according to the CLSI EP-6P protocol indicated no significant deviance from linearity ($F = 0.22$; $p = 0.80$). In the autism group, mean (SD) 24-h urinary 5-HIAA excretion [0.0165 (0.0086) mmol/24 h or 1.74 (0.79) mmol/mol creatinine] was similar to that

reported previously for control groups and autistic patients (Launay et al., 1987; Minderaa et al., 1987; Anderson et al., 1989; Deacon, 1994).

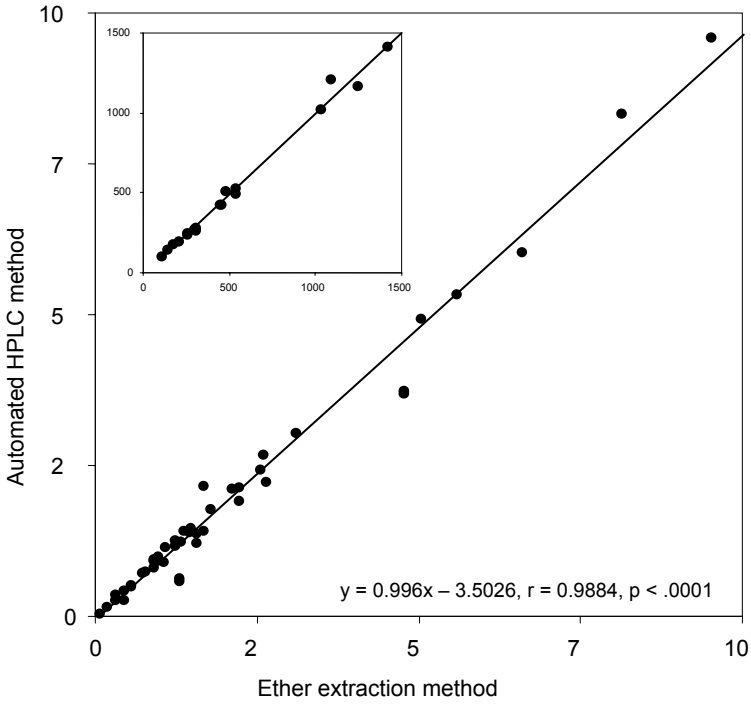


Figure 4.3: Comparison of 5-HIAA results for 63 urine samples as derived from the automated SPE-HPLC method (y axis) and the ether extraction method (x axis). The main panel shows values from 0 to 100 µmol/L. The inset shows values from 100 to 1500 µmol/L.

Discussion

A variety of methods for urine 5-HIAA analysis have been described, including several HPLC methods (Deacon et al. 1994). The automated SPE-HPLC method we developed reduced the per sample analysis time considerably, with a concomitant increase in sample throughput. The CVs for this method were ~50% lower than those for the manual method. We found that use of the on-line SPE system allows regeneration of SPE cartridges up to 40 times when small amounts of urine or plasma are extracted (Kema et al., 2001). This can be done with negligible loss of accuracy. During the 2-year period that this method has been in operation, interferences from drugs and other substances have been extremely rare (and discernable). The group mean urine concentrations and excretion rates reported here for 5-HIAA were within the reference intervals established at the University Medical Center Groningen and were in excellent agreement with previous reports (Launay et al., 1987; Minderaa et al., 1987; Anderson et al., 1989; Deacon, 1994).

In summary, our automated SPE-HPLC method for the determination of urinary 5-HIAA appears to offer advantages in time and precision compared with manual chromatographic methods.

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