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A New High Performance Liquid Chromatography (HPLC) Method for the Quantitation of Strychnine in Urine and Tissue Extracts

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Summary: A high performance liquid chromatography (HPLC) method was developed for the quantitation of strychnine in urine of children with nonketotic hyperglycinaemia and other developmental disorders treated with the alkaloid. Mobile and stationary phases were polar, i.e. methanol-water-330 g/kg ammonia (volumes, 85 ml + 14.2 ml + 0.8 ml) and LiChrosorb Si-60, 7 μm. Brucine was the internal standard. Extraction was performed by the Extrelut technique. At strychnine nitrate concentrations in urine of 21, 126, and 760 μg/l, recovery was 92.1 ± 8.7, 98.1 ± 2.7, and 102.5 ± 2.7%. A child with nonketotic hyperglycinaemia under continued strychnine treatment excreted 1 to 13.6% of the daily dose unmetabolized in urine. The method was also suitable for the estimation of unreacted strychnine in tissue extracts. The fast disappearance in vitro of strychnine from a guinea pig liver preparation was confirmed.

Eine neue Methode für die quantitative Bestimmung von Strychnin in Harn und Gewebsextrakten mit Hochleistungsflüssigkeitschromatographie (HPLC)

Zusammenfassung: Zur Bestimmung von Strychnin im Harn von Kindern mit nicht-ketotischer Hyperglycinämie und anderen Entwicklungsstörungen, die mit dem Alkaloid behandelt werden, wurde eine Methode unter Verwendung der Hochleistungsflüssigkeitschromatographie (HPLC) entwickelt. Mobile und stationäre Phase waren polar, es wurden Methanol/Wasser/330 g/kg Ammoniak (Volumina, 85 ml + 14,2 ml + 0,8 ml) und LiChrosorb Si-60, 7 μm, verwendet. Brucin diente als innerer Standard. Die Extraktion wurde mit der Extrelut-Technik durchgeführt. Die Wiederfindung für Strychninnitrat-Konzentrationen im Harn von 21, 126 und 760 μg/l betrug 92,1 ± 8,7, 98,1 ± 2,7 und 102,5 ± 2,7%. Ein Kind mit nicht-ketotischer Hyperglycinämie schied unter kontinuierlicher Strychninbehandlung 1–13,6% der täglichen Dosis unverändert im Harn aus. Die Methode erwies sich ebenso als geeignet für die Bestimmung von unverändertem Strychnin in Gewebsextrakten. Das schnelle Verschwinden von Strychnin aus einer Meerschweinchenleber-Präparation in vitro wurde bestätigt.

Introduction

Strychnine has a long history as a spinal analeptic but little is known of its metabolism. If given in repeated toxic doses to cats, dogs and guinea pigs, only a small amount is excreted in biologically active form in the urine, all of it within 24 h after the last dose (1). Unreacted strychnine is found in most tissues of cats and dogs and it is concentrated 6–7 fold in liver and kidney (2). In various animal species, the bulk of the drug is metabolized in the liver (3, 4), and it is not metabolized in gut, muscle, heart, kidney, brain or blood (4). In vitro transformation of strychnine by rabbit liver, measured with the methyl orange reaction, was apparently enzymatic; it occurred in microsomes enriched with soluble fraction, required O_2 and NADPH, and had a narrow pH optimum at 8.4 (4). Phenobarbital and other inducing compounds given to rats and rabbits enhanced strychnine metabolism and lowered sensitivity (5, 6). Male rats had higher tolerance to strychnine than females (7). Of at least 4 metabolites produced by rabbit liver homogenates, only one was identified as 2-hydroxystrychnine which in mice had a toxicity of only one hundredth of the parent alkaloid (6). Glucuronidation and sulfatation played only a minor role, if any, in the detoxification of strychnine by rabbit microsomes (4).

A few years ago, we initiated the treatment of children with nonketotic hyperglycinaemia and other developmental disorders with pharmacological doses of strychnine (8–11). Thus there arose the need to measure unreacted strychnine in urine and other biological fluids. Colour reactions (12), measurement of absorbance at one or two wavelengths after separation (13–15), double labeling techniques (16), GLC separation (17), and existing HPLC methods for grain baits (18) and drug

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mixtures (19, 20) were unsatisfactory in one or more respects. The interesting reversed phase HPLC method of *Crouch & Short* (21) did not separate strychnine from brucine except by use of a solvent gradient. We developed a HPLC method which differs from the above (21) by making use of polar stationary and mobile phases and an isocratic solvent with brucine as the internal standard. Here we describe the procedure and its first applications.

Materials and Methods

Strychnine nitrate Ph. Helv. VI (Siegfried, Zofingen, Switzerland) (M_r free strychnine 334.4; M_r strychnine nitrate 397.4); brucine • $2H_2O$ p.a. (M_r free brucine 394.4; M_r brucine • $2H_2O$ 430.4), aqueous ammonia 33% (d = 0.885), hexane p.a., and tetrahydrofuran Uvasol (Merck, Darmstadt, F.R.G.). Extrelut® for column extraction (Merck, F.R.G.). Equipment for HPLC: pump Altex 100 (Altex Scientific Inc., Berkeley Ca., USA), injector UGK and detector at 254 nm model 440 (Waters Associates Inc., Milford Ma., USA). HPLC column (46 × 250 mm) and precolumn (4.6 × 40 mm) were packed with LiChrosorb Si-60, 7 µm (Knauer, Berlin F.R.G.), the *stationary phase*. The *mobile phase* was methanol-water-330 g/kg ammonia (volumes, 85 ml + 14.2 ml + 0.8 ml).

Extraction

A sample of 4 ml urine was spiked with 100 µl internal standard solution (brucine • $2H_2O$, 20 mg/l in methanol), adjusted to pH 12.0 with 3 mol/l NaOH and applied to a column (9 cm × 2.5 cm) containing 4 g Extrelut. The sample was allowed to equilibrate with the Extrelut for 10 min. Substances were eluted with 50 ml dichloromethane and the total eluate was evaporated to dryness in vacuo. The residue was dissolved in 2 × 0.5 ml methanol, transfered to a small test tube and the methanol evaporated in a stream of nitrogen.

Chromatography

For HPLC the residue was dissolved in 50 µl of mobile phase, and 1–5 µl were injected. The flow rate was 2.0 ml/min. The elution was monitored at 254 nm with a detector sensitivity of 0.005 absorbance units on the full scale and a recorder sensitivity of 5 mV. The column was not stored in the mobile phase which was polar and basic. Instead, the column was rinsed with approx. 30 ml of tetrahydrofuran followed by hexane as an inert solvent for storage. Before it was reused, the column was rinsed with at least 10 ml of tetrahydrofuran followed by the mobile phase. By this treatment, the column was kept clean and could be used successfully and extensively for at least two months. The small dead volume which appeared at the top of the column bed after several months of use was filled with Li-Chrosorb Si-60 and the column employed further. (Theoretically one could avoid the formation of a dead volume by inserting a silica gel precolumn between pump and injector).

Calculation

The concentration of strychnine in the sample was calculated using the formula:

$$c_{\text{strychnine}} = R \cdot F \cdot c_{\text{brucine} \cdot 2H_2O}$$

where R was the peak height ratio, strychnine to brucine, at 254 nm. Factor F was determined previously using the standard substances:

$$F = \frac{1}{R} \cdot \frac{c_{\text{strychnine}}}{c_{\text{brucine} \cdot 2H_2O}}$$

Under our conditions it was 0.40 for free strychnine.

Results and Discussion

Extraction and quantitation of strychnine

For *extraction* of urines, solvents with higher polarity such as dichloromethane-isopropanol (volumes, 90 ml + 10 ml) or ethyl-acetate offered the advantage of smaller volumes, but they resulted in tailings of UV absorbance ("solvent peaks"), which interfered with the strychnine peak. With toluene or benzene as the extraction solvents, the biological background was almost nil but brucine recovery was variable. The Extrelut technique (22) provided for excellent recovery and low biological background. High urinary salt concentrations (up to 0.3 mol/l sodium chloride added) did not impair the extraction.

For *high performance liquid chromatography*, several solvents were tried as mobile phases, e.g. diethylether-methanol-diethylamine (20) and dichloromethane-methanol-diethylamine mixtures. With methanol-H_2O-330 g/kg ammonia (volumes, 85 ml + 14.2 ml + 0.8 ml), optimal resolution and the clearest background were obtained (number of theoretical plates 900–1200). Several unspiked urines from healthy children and adults were processed, and peaks interfering with the position of strychnine and brucine were not observed (fig. 1b).

In order to evaluate the precision of the method, strychnine nitrate was added to a urine specimen of a control person at a concentration of 220 µg/l, and 4 ml portions were processed. The variation coefficient (VC) was 0.8% (n = 12).

A linear calibration curve was obtained by the addition of strychnine nitrate to urine to yield concentrations of 20–840 µg/l (coefficient of correlation r = 0.999; n = 12). The calibration curve matched the curves obtained from pure standards.

Recovery was tested by the addition of strychnine nitrate to control urines of 6 children at low (21 µg/l), intermediate (126 µg/l) and high (760 µg/l) concentrations. Recovery was 92.1 ± 8.7, 98.1 ± 2.7, and 102.5 ± 2.7%, respectively.

The detection limit was 2–4 ng per injection; starting with a 4 ml urine sample, this corresponded to a strychnine concentration of 5–10 µg/l. When the urinary strychnine concentration exceeded 200 mg/l, sample size could be reduced.

Strychnine metabolites were not investigated in our study. It can be expected that they will not interfere with the measurement of strychnine. Because of their different polarity and molecular weight, they will either not be extracted or be separated from strychnine chromatographically.

Application of the method

Chromatograms of strychnine extracted from the urine of a child treated with the alkaloid and from a spiked

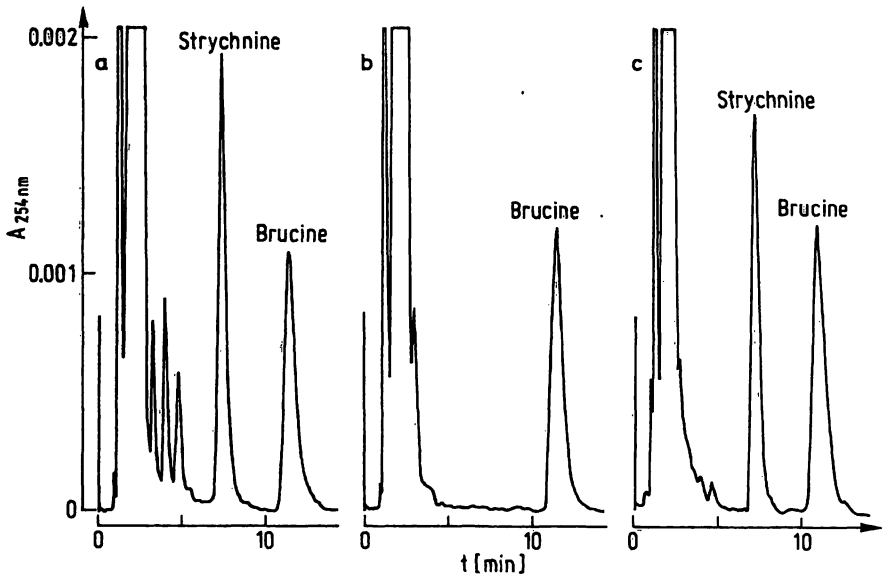

Fig. 1. HPLC of strychnine in human urine (a, b) and in guinea pig liver extract (c).
Brucine · 2 H_2O (500 µg/l) was the internal standard. Column (46 × 250 mm) and pre-column (4.6 × 40 mm) of LiChrosorb Si-60, 7 µm; mobile phase methanol-water-330 g/kg ammonia (volumes, 85 ml + 14.2 ml + 0.8 ml); flow rate 2 ml/min
a) Urine of patient B.F.; injected volume: 3 µl of extract, equivalent to 0.24 ml of urine.
b) Urine of a healthy control person without strychnine (otherwise same as a).
c) Supernatant of guinea pig liver extract after incubation; injected volume: 4 µl of extract, equivalent to 0.32 ml of diluted mixture (see fig. 2).

control urine are shown in figures 1a and 1b. Patient B.F. (8) suffering from nonketotic hyperglycinaemia and treated with daily oral strychnine nitrate for 3 years excreted from 1 to 13.5% of the drug unmetabolized (tab. 1). There was no correlation between the proportion of unmetabolized strychnine in urine and the duration of therapy or the administered dose. It is interesting that these results were in good agreement with those obtained many years ago in man (3) and in animals (1).

The method was also applied to tissue extracts (fig. 1c). An experiment published by *Adamson & Fouts* (4) was duplicated and the disappearance of unreacted strychnine in a guinea pig liver microsome preparation followed (fig. 2). Strychnine concentration fell in a first order mode to one tenth of the initial concentration at a rapid rate (50% disappearance in 16 min). The suitability of the method for the quantitation of strychnine in tissue extracts was thus established.

Tab. 1. Excretion of strychnine in 24 h urine of patient B.F. with nonketotic hyperglycinaemia (8) during continued treatment with oral strychnine nitrate.

Age	Weight	Dose of strychnine nitrate	Strychnine excreted	Percent of administered strychnine in 24 h-urine
(a)	(kg)	(mg/d)	(µg/24 h)	
6/12	7.5	1.2	18.9 *19.9	1.9
7/12	7.8	3.2	29.3 *23.7	1.0
7/12	8.0	1.8	26.3 *18.3	1.5
8/12	8.9	3.0	116.3 *112.0	4.6
3	11.5	12.8	335.0	3.1
3½	12.1	12.8	1475.0	13.6

* Values obtained from a second extraction and chromatography done after the samples had been kept at – 20 °C for 1 year.

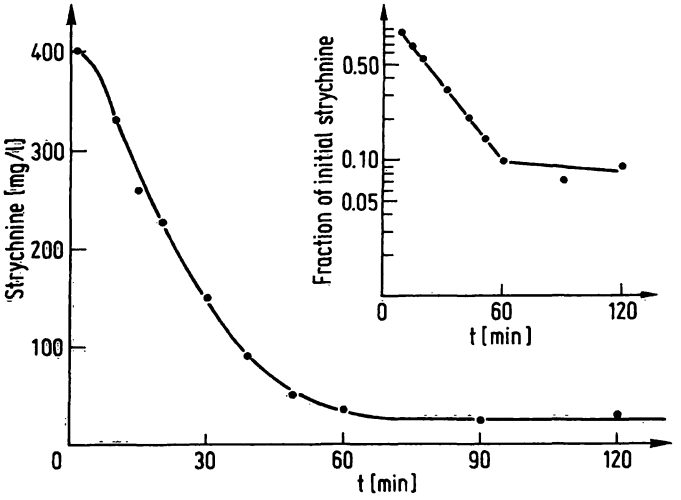

Fig. 2. Metabolism of strychnine by guinea pig liver microsomal preparation in vitro.
Supernatant, 9000 *g*, from 1.2 g of liver (1:4 homogenate in 0.15 mol/l KCl) was incubated at 37 °C in a *Warburg* apparatus under oxygen with 12 µmoles of strychnine nitrate in 0.12 mol/l Tris-HCl pH 8.2. Initial concentrations of cofactors were: nicotinamide 40 mmol/l, NADP 55 µmol/l, glucose-6-phosphate 5 mmol/l. Volume was 12 ml. At the times indicated, 0.6 ml aliquots were withdrawn, denatured for 2 min at 100 °C, centrifuged, diluted with water 800 times, and 4 ml of diluted mixture were processed for strychnine extraction and chromatography.

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