Journal of Chromatography, 226 (1981) 175—182
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 992

DETERMINATION OF THERAPEUTIC PLASMA CONCENTRATIONS OF TETRABENAZINE AND AN ACTIVE METABOLITE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 23rd, 1981; revised manuscript received June 15th, 1981)

SUMMARY

A reversed-phase high-performance liquid chromatographic method for the determination of tetrabenazine and a hydroxy metabolite in plasma is described. Tetrabenazine and the hydroxy metabolite are quantified as their dehydro derivatives using fluorescence detection. This method has been applied to the analysis of plasma samples from patients with Huntington's chorea and has been found to be sensitive, reliable and specific for tetrabenazine and the hydroxy metabolite. The plasma concentrations of tetrabenazine found in patients were lower than could be detected using previously published methods.

INTRODUCTION

Tetrabenazine (TB) is currently used to treat the abnormal movements of Huntington's chorea and other disorders [1]. Although its urinary metabolites have been described [2] little is known of its pharmacokinetics in man.

In order to study relationships between dose, plasma levels and therapeutic effect of TB, a reliable and sensitive method to estimate the plasma concentrations of TB and its metabolites is necessary. Quinn et al. [3] have described a spectrofluorometric method for the determination of TB in plasma and tissues. This method detects levels of 2—5 μg in plasma and relies on the extraction procedure for specificity. The sensitivity of the method was improved by a derivatization step [4], the limit of sensitivity being between 30 and 200 ng per g of biological material. This method does not appear to have excluded interference by metabolites. In the application of these methods to quantifying the distribution of TB in animal tissues [3, 5], no differentiation between the concentrations of TB and any of its metabolites in these tissues was attempted.

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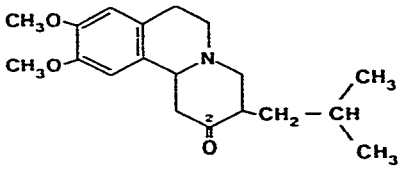
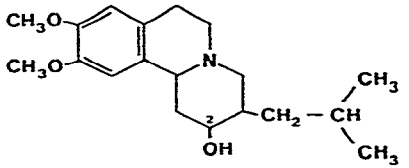


TETRABENAZINE



HYDROXYTETRABENAZINE

Fig. 1. Structures of tetrabenazine and its metabolite, hydroxytetrabenazine.

Nine metabolites of TB have been detected in urine after its administration to man [2].

This paper describes a reversed-phase, high-performance liquid chromatographic (HPLC) method for the determination of TB and a hydroxy metabolite (HTB, Fig. 1) in plasma. This method has been applied to the analysis of plasma samples from patients with Huntington's chorea and has been found to be sensitive, reliable and specific for TB and HTB. Plasma concentrations of TB found in patients were well below levels detectable by previous methods [3, 4].

EXPERIMENTAL

Standards and reagents

Tetrabenazine (1,3,4,6,7,11*b*-hexahydro-3-isobutyl-9,10-dimethoxy-2H-benzo[*a*]quinolizin-2-one) and the *cis* and *trans* isomers of the 2-hydroxy derivative of tetrabenazine (HTB) were kindly supplied by Roche Products (Sydney, Australia).

The mercuric acetate reagent used for the derivatization of TB and HTB to their dehydro derivatives [2] consisted of 2.0% mercuric acetate in pH 4 acetate buffer [96 ml glacial acetic acid, 32 ml sodium hydroxide (40%) and 4 ml distilled water] [4].

Acetonitrile, methanol (specially purified for HPLC) and PIC B-5 (pentanesulphonic acid) were supplied by Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were reagent grade.

Instrumentation

Reversed-phase HPLC was performed using a Waters Model M45 solvent delivery system, a U6K universal injector, a Phenyl μBondapak column and a C_{18} μBondapak column (each 30 × 3.9 mm I.D., 10 μm average particle size). A guard column packed with Bondapak C_{18}/Porasil B was used in all studies. The absorbance of the eluent was determined using a Waters Model 450 variable-wavelength UV absorption detector. The fluorescence of the eluent was monitored using a Schoeffel PS970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.).

Liquid chromatography

The influence of the following parameters on the chromatography of TB and its dehydro derivative were examined: pH, buffer composition, acetonitrile concentration, methanol concentration, addition of PIC reagents to mobile phase and support type.

Thin-layer chromatography

Since authentic samples of most TB metabolites were not available, thin-layer chromatography (TLC) of urine extracts from patients on TB therapy was carried out to identify and isolate individual metabolites. Urine (50 ml) was concentrated to 10 ml with a rotary evaporator (Büchi, Flawil, Switzerland) made to pH 5.2 with 1 ml 1 *M* acetate buffer and incubated at 37°C overnight with 0.5 ml ext. Helix pomatia (Boehringer, Mannheim, G.F.R.) to hydrolyze glucuronide conjugates. This solution was then adjusted to pH 9.6 with 5 *M* sodium hydroxide and TB and its metabolites extracted into 25 ml ethyl acetate by vortexing for 30 sec. After centrifuging at 1500 g for 10 min, the ethyl acetate layer was removed and evaporated to dryness. The residue was redissolved in 2 ml dichloromethane and spotted on a 0.25 mm thick silica gel plate (Kieselgel G, Merck, Darmstadt, G.F.R.). The plate was developed with a chloroform—*n*-butanol—2.5% ammonia solution (80:20:0.6) system and spots were visualized by converting TB metabolites to their dehydro compounds with mercuric acetate reagent [2]. Spots were scraped off and extracted in 2 ml methanol. The methanol was evaporated to dryness under nitrogen and the residue dissolved in 1 ml 0.25 *M* sulphuric acid prior to HPLC.

Plasma assay

Procedure. Plasma samples were processed by transferring 2 ml into a glass centrifuge tube containing 1 ml 0.1 *M* carbonate buffer (pH 9.6), and 2.5 ml diethyl ether were added. After the sample was vortexed for 20 sec it was centrifuged for 10 min at 1500 *g*. The lower aqueous phase was snap-frozen with acetone—dry ice and the ether decanted into a calibrated 3-ml glass centrifuge tube. The ether was evaporated to about 1 ml in a 40°C water bath prior to the addition of 100 µl 0.5 *M* sulphuric acid. This mixture was then vortexed for 15 sec and centrifuged for 5 min at 1500 *g*. The lower aqueous phase was snap-frozen with acetone—dry ice and the upper ethereal layer discarded. The aqueous phase was mixed with 100 µl mercuric acetate reagent, heated in a boiling water bath in an all-glass stoppered centrifuge tube for 30 min and cooled. The tubes had been tared and were made to weight with distilled water after heating. An aliquot (20 µl) of this solution was then injected onto the liquid chromatographic system consisting of a C_{18} µBondapak column and fluorescence detector. The mobile phase was acetonitrile—1% acetate buffer, pH 4.5 (50:50) at a flow-rate of 1 ml/min. The fluorescence of the eluent was quantified using an excitation wavelength of 265 nm and an emission filter (KV418).

Quantitation. The procedure was standardized by analyzing drug-free plasma samples spiked with known amounts of the analytes. Calibration curves were established by plotting absolute peak heights of analytes for a given injection

volume against plasma analyte concentration. Standards were run each day to control changes in detector response.

Recovery. Recoveries from plasma were estimated by a comparison of peak heights obtained from the injection of known quantities of the analytes in aqueous solutions treated with mercuric acetate reagent and known quantities added to plasma before extraction and derivatization.

Reproducibility. Within-day precision was determined by performing replicate analyses of spiked plasma samples.

Specificity. The specificity of the plasma assay for TB and HTB was verified by injection of other TB metabolites which had been separated by TLC and by processing plasma samples from patients on various medications.

RESULTS AND DISCUSSION

Chromatographic behaviour and fluorescence of tetrabenazine

Preliminary studies on the chromatographic behaviour of TB were carried out with a Phenyl μBondapak column and UV absorbance detection. TB at a concentration of 3 μg/ml gave a good symmetrical peak at 282 nm after 7 min with a methanol—0.01 *M* ammonium hydrogen phosphate, pH 6.5 (60:40) system. However, no detectable concentrations of TB were found in plasma extracts from patients on steady-state TB therapy for Huntington's chorea when the eluent was monitored for absorbance. With this mobile phase, TB showed no fluorescence. Fluorescence of TB was only significant in an acidic mobile phase. Unfortunately, with the various acidic mobile phases tried, tetra-

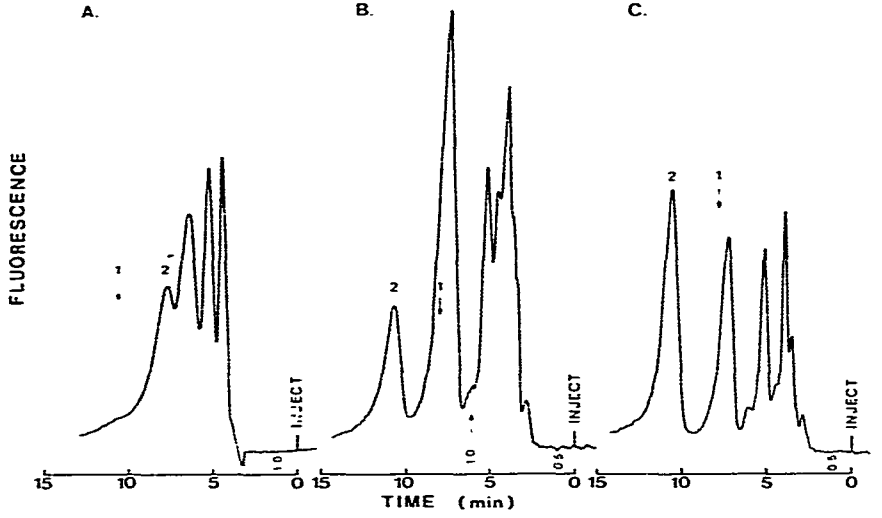


Fig. 2. Chromatograms of urine extracts from a patient being treated with TB. Urine was extracted as described for thin-layer chromatography, but heated with mercuric acetate reagent before HPLC. Mobile phase and λ_{ex}: (A) methanol—1% acetate buffer, pH 4.0 (60:40), 310 nm; (B) acetonitrile—1% acetate buffer, pH 4.5 (50:50), 315 nm; (C) acetonitrile—1% acetate buffer, pH 4.5 (50:50), 265 nm. (1) shows the retention of DTB in each system (TB was not found in urine) and (2) shows the position of DHTB. Fluorescence range is shown in μA, and the arrow marks a change.

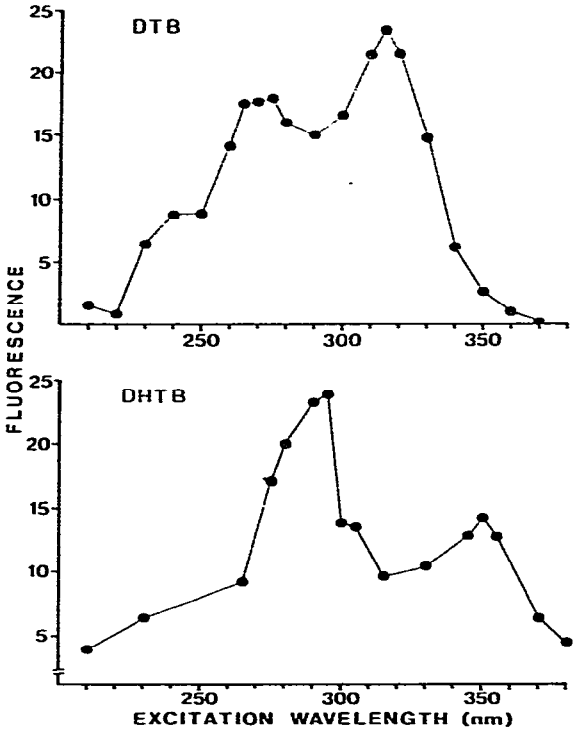


Fig. 3. Excitation spectra of DTB and DHTB. Each point was obtained by injecting a 2-ng sample onto the HPLC system.

benazine did not resolve sufficiently from the solvent front for quantitative studies.

Satisfactory fluorescence and column retention could only be achieved after derivatization of tetrabenazine. TB and HTB form highly fluorescent dehydro derivatives (DTB and DHTB) when heated in the presence of mercuric acetate [4]. Maximum fluorescence was observed after heating with mercuric acetate in a boiling water bath for 30 min. Heating of HTB for 40 min (or longer) resulted in a diminished fluorescence of DHTB and an extraneous peak on the chromatogram.

The composition of the mobile phase was found to be critical for the separation of DTB and DHTB (Fig. 2A, B). The lower excitation maximum for DTB (265 nm, Fig. 3) was used to reduce the potential interference from a urinary metabolite of TB (Fig. 2C), which was observed at 310 nm (Fig. 2B). In all HPLC systems used, we were unable to resolve the two stereoisomers of DHTB. They also had the same fluorescence intensity over all excitation wavelengths (Fig. 3). Maximum fluorescence intensity for DHTB was found at 305 and 350 nm, but satisfactory sensitivity was present at 265 nm.

Plasma assay

Fig. 4 shows chromatograms of blank plasma and plasma from a patient on TB therapy. The retention times for DTB and DHTB were 7.0 and 9.7 min, respectively. Approximately 0.1 ng/ml of TB and 1 ng/ml of HTB in plasma

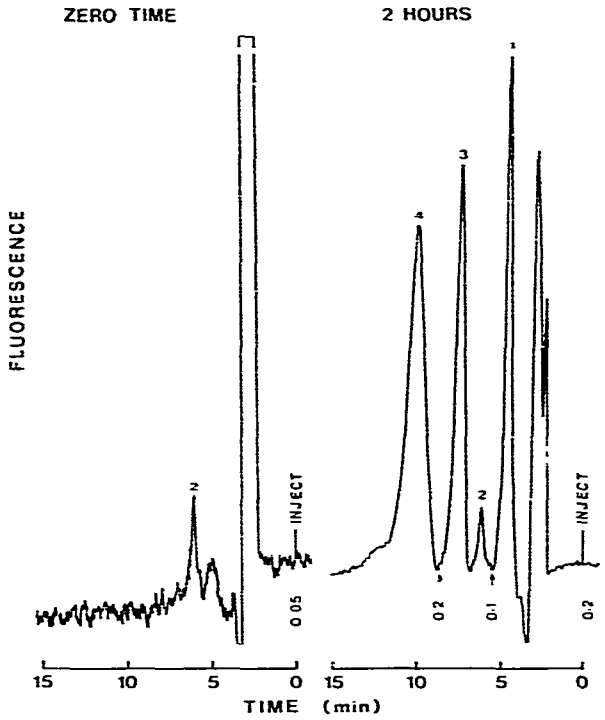


Fig. 4. Chromatograms of extracts of plasma taken before (zero time) and 2 h after a single oral dose of TB (50 mg). Fluorescence range is shown in μA, and changes are marked with an arrow. Peaks: (1) metabolite(s) of TB; (2) plasma; (3) DTB; (4) DHTB.

could be detected. Other metabolites of TB found on thin-layer plates eluted together (Fig. 4) and could not be quantified.

A number of potential internal standards were evaluated for the plasma assay. Unfortunately, a substance having both a desirable retention time and an acceptable fluorescence emission was not found. Plasma from patients taking a variety of medications (Table I) were found not to contain any substances which interfered with the assay.

Standard curves of the fluorescence plotted against plasma concentrations for DTB and DHTB gave correlation coefficients of 0.998 and 0.997, respec-

TABLE I

DRUGS TESTED FOR INTERFERENCE

Amiloride	Hydrochlorothiazide
Amoxycillin	Methyldopa
Bendrofluazide	Nitrazepam
Chlorothiazide	Potassium chloride
Digoxin	Prednisolone
Ferrous sulphate	Propranolol
Folic acid	Pyridoxine
Furosemide	Salicylate
Glyceryl trinitrate	Vancomycin
Heparin sulphate	

tively. The good linearity of response was found to be consistently reproducible for each set of standards prepared. The precision of the assay is given in Table II. For both TB and HTB the coefficient of variation was less than 5%. The recoveries of TB and HTB from plasma after extraction were 74% and 65%, respectively.

TABLE II

REPRODUCIBILITY OF ASSAY

	Concentration (ng/ml)	Mean	*n*	S.D.	C.V. (%)
Tetrabenazine	2.5	4.85	8	0.20	4.1
	10.0	18.98	10	0.51	2.7
Hydroxytetrabenazine	10.0	26.34	10	0.64	2.4

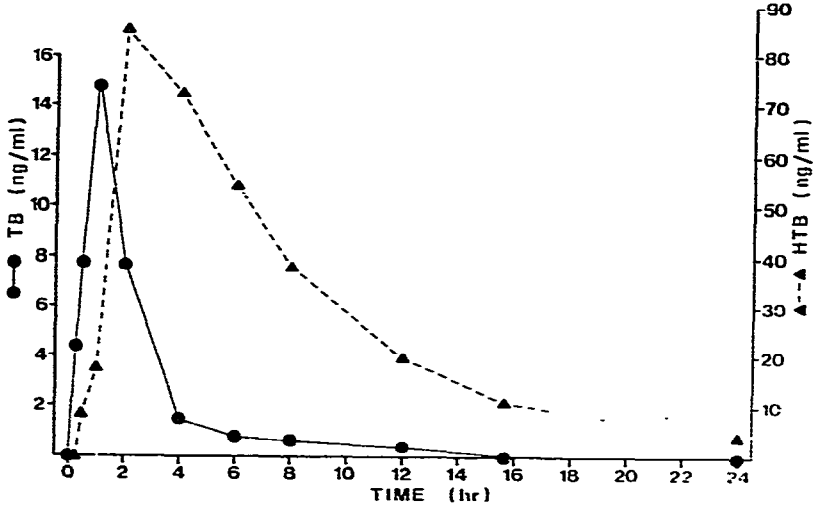


Fig. 5. Plasma concentrations of TB and HTB following a single 50-mg oral dose administered to a fasting patient.

The application of the present method to the analysis of TB and HTB in the plasma of a patient given a single 50-mg dose of TB is shown in Fig. 5. It is observed that the plasma concentrations of TB are lower and present for a shorter period of time after dosing than those of HTB. These high levels of HTB may be clinically significant because HTB is known to have brain amine depleting activity similar to that of TB [6]. These high concentrations of HTB also demonstrate the need for an assay for TB and HTB which is not only sensitive but also specific.

ACKNOWLEDGEMENTS

We acknowledge support for this work by Roche Products Pty. Ltd. (Sydney, Australia), and technical assistance by Miss E. Symons. We are grateful to Miss H. Lawler for typing the manuscript.

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