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High-performance liquid chromatographic method using ultraviolet detection for measuring metrifonate and dichlorvos levels in human plasma

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ABSTRACT

We report high-performance liquid chromatographic methods using ultraviolet detection, developed for the first time in our laboratory with sensitivity to detect clinically significant concentrations of metrifonate (MTF), an experimental drug for Alzheimer disease, and its active anticholinesterase metabolite, dichlorvos (DDVP). The determination limit of the method for MTF and DDVP was 1 μg/ml and 40 ng/ml, respectively. Stability of MTF and DDVP at various temperatures in water, buffered solutions and in human plasma were also studied.

INTRODUCTION

Metrifonate, O,O-dimethyl (1-hydroxy-2,2,2-trichloroethyl)phosphonate (MTF), is an organophosphorus compound and has been used since 1960 in the treatment of schistosomiasis [1,2]. It has been shown that this drug yields the direct-acting irreversible cholinesterase (ChE) inhibitor, dichlorvos (2,2-dichlorovinyl dimethyl phosphate, DDVP) *in vivo* [3,4]. Animal studies have shown that MTF is more powerful and less toxic than physostigmine at high doses (80 mg/kg) [5]. Long-acting ChE inhibitors have potential use as therapeutic agents for Alzheimer disease (AD). Becker *et al.* [6] have reported that MTF may have advantages over other currently available ChE inhibitors for the treatment of AD.

The pharmacokinetic and pharmacodynamic properties of drugs can be understood only if sensitive analytical methods are available to measure drug levels after dosing. The quantitation of MTF and DDVP has been achieved from human tissues only by gas chromatography–mass spectroscopy (GC–MS) using deuterium-labeled MTF and DDVP as internal standards [7] due to the decomposition of MTF to form DDVP at high temperature. Nordgren [7] used liquid–liquid extraction followed by evaporation to dryness, to isolate both MTF and DDVP. DDVP is a liquid at room temperature and therefore any isolation procedure which involves evaporation may result in a loss of DDVP. Moreover, GC–MS methods that require deuterium-labeled compounds will not be economical for routine monitoring of patient plasma.

Here, we report, for the first time, sensitive high-performance liquid chromatographic (HPLC) methods using ultraviolet (UV) detection for the determination of MTF and DDVP levels in human plasma.

EXPERIMENTAL

Drugs and chemicals

MTF (98% pure) and DDVP (99% pure) were from Chem Service (West Chester, PA, USA). Sodium salt of octanesulfonic acid (OSA), meth-

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anol, tetrahydrofuran of HPLC grade and all other chemicals of reagent grade were from Fisher Scientific (St. Louis, MO, USA).

Apparatus and chromatographic conditions

The HPLC system consisted of a PM-30A dual-piston pump, a Rheodyne 7125 injector with a 200-μl sample loop (both from Bioanalytical System, West Lafayette, IN, USA), a Model 481 LC spectrophotometer from Water Assoc. (Milford, MA, USA) and a Model 4270 integrator from Spectra-Physics (San Jose, CA, USA).

Chromatography was carried out at room temperature using a 10 μm particle diameter C_{18} column (30 cm × 3.9 mm I.D.) and a guard column packed with C_{18}/Corasil (37–50 μm) (both from Waters Assoc.). The UV detector was set at 210 nm. The mobile phase composition for MTF determination was 1 m*M* OSA in 10% (v/v) methanol and 0.1% (v/v) tetrahydrofuran (pH 3.0) and for DDVP it was 1 m*M* OSA in 30% (v/v) methanol (pH 3.0). The mobile phase was filtered under vacuum through a 0.45-μm Nylon 66 filter (Rainin Instrument, Woburn, MA, USA) and sonicated to remove air bubbles. The flow-rate was 2.0 ml/min for MTF and 1.5 ml/min for DDVP determination.

Source of plasma

Human plasma (pH 7.0) from normal volunteers (obtained from the blood bank at St. Johns Hospital in Springfield, IL, USA) or from AD patients, prior to and 30 min after MTF dosing (7.5 mg/kg, orally) [8,9] was stored at −70°C until analysis. Prior to extraction, plasmas were thawed on ice and brought to 4°C.

Extraction procedure for MTF determination

Human plasma (1 ml) and chloroform (5 ml) were combined in a glass screw-cap test tube, vortex-mixed for about 15 s, centrifuged (800 *g*, 15 min, 0–5°C) in an IEC Centra-7R centrifuge from Fisher Scientific. After centrifugation, 4.5 ml of the chloroform layer were transferred to a screw-cap test tube containing 3.5 ml of 5 *M* hydrochloric acid. The mixture was vortex-mixed for 15 s, centrifuged (800 *g*, 15 min, 0–5°C) and the chloroform layer (4 ml) was transferred to another screw-cap test tube containing 200 mg of dried-rite. The mixture was vortex-mixed and centrifuged (800 *g*, 5 min, 0–5°C). The chloroform layer (3.8 ml) was transferred to an acid-washed 5-ml conical-bottom glass vial and evaporated under a gentle stream of nitrogen at room temperature. The residue was reconstituted with 120 μl doubly distilled water, vortex-mixed and centrifuged (11 000 *g*, 5 min, 0–5°C). A 100-μl aliquot of the supernatant was then injected into the chromatographic column.

Sample pretreatment for DDVP determination

Human plasma (400 μl) and hydrochloric acid (80 μl, 6 *M*) were pipetted into a polycarbonate test tube and vortex-mixed for about 1 min. The mixture was centrifuged (39 000 *g*, 30 min, 0–5°C using a Sorvall RC-5B refrigerated centrifuge from DuPont Instruments, Newtown, CT, USA) and 400 μl of the supernatant were transferred to an Eppendorf tube and kept frozen at −70°C until analysis. Prior to injection, samples were brought to room temperature, centrifuged (11 000 *g*, 5 min, 0–5°C), the supernatant was filtered (RC58 filter, 0.2 μm from Schleicher and Schuell, Keene, NM, USA) (3000 *g*, 15 min, 0–5°C) and a 200-μl aliquot was injected into HPLC column.

Calibration curves

Standard solutions of MTF (0.5–5 μg) and DDVP (15–300 ng) in water were injected into the chromatographic column, and the peak areas for MTF and DDVP were measured.

To plasma samples MTF and DDVP were added to final concentrations ranging from 1 to 10 μg/ml or 0.15 to 1.5 μg/ml respectively, and extracted as described above.

Stability studies

MTF and DDVP were dissolved in doubly distilled water (kept at 4, 22 and 37°C) and in 0.01 *M* sodium acetate buffer solutions (kept at 37°C, pH 1, 4, 7 and 11) to obtain a concentration of 1 mg/ml for MTF and 30 μg/ml for DDVP. Aliquots were removed at various intervals and injected into HPLC column.

To plasma equilibrated at various temperatures (4, 22 and 37°C), MTF and DDVP were added to obtain a final concentration of 40 μg/ml

for MTF or 5 μg/ml for DDVP. Aliquots were removed at various times upto 4 h and kept at −70°C until analysis.

RESULTS

Two different HPLC methods with UV detection were developed for the determination of MTF (retention time 10.8 min) and its active metabolite, DDVP (retention time 12.6 min). The method employed for MTF determination also detected DDVP (retention time of MTF 10.8 min, that of DDVP 44.2 min) and *vice versa* (retention time of MTF 7.5 min, that of DDVP 12.6 min) but contaminating peaks coeluting from plasma after extraction made it necessary to use two different methods to quantitate these two compounds from human plasma.

Peak areas were used as a measure of MTF and DDVP concentration. The within- and between-day coefficients of variation (C.V.) for standard injections were less than 4.04% (n = 26) and 3.71% (n = 17) for MTF and 2.94% (n = 12) and 3.22% (n = 44) for DDVP.

Human plasma from normal volunteers or pretreatment plasma from AD patients was subjected to MTF and DDVP isolation procedures; chromatograms showed no peak at the retention times of MTF and DDVP. Figs. 1 and 2 show the chromatograms obtained from plasma of an AD patient, 30 min after MTF dosing (7.5 mg/kg, orally). The recovery of MTF and DDVP from normal volunteer plasma spiked with varying concentrations of MTF and DDVP is given in Table I; a plot of theoretical values *versus* experimentally determined amount (based on calibration curve generated with spiked plasma samples) gave slope and y-intercept values of 0.30 and 0.06 μg/ml for MTF and 0.76 and 37.34 μg/ml for DDVP. The determination limit of the method was 1 μg/ml for MTF and 40 ng/ml for DDVP.

Metrifonate was stable in plasma at 4°C upto 4 h, but at 22°C it slowly degraded to about 25% of

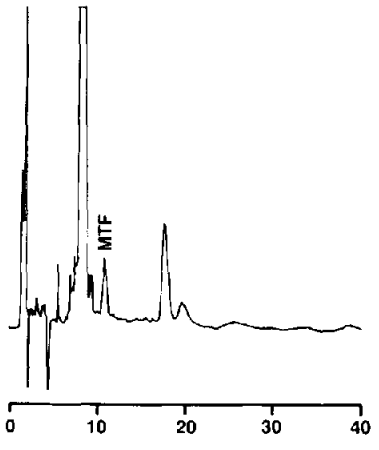

Fig. 1. Chromatogram of plasma from an AD patient (30 min after 7.5 mg/kg MTF, orally) after MTF extraction.

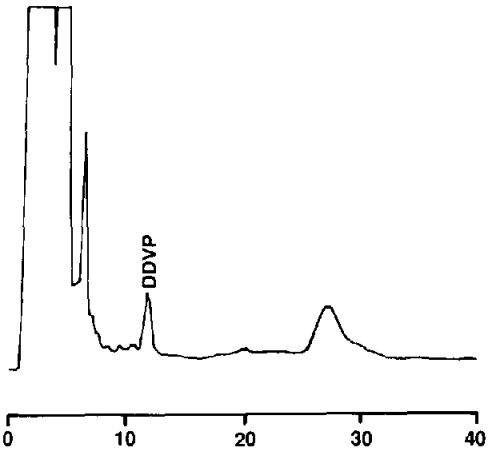

Fig. 2. Chromatogram of plasma from an AD patient (30 min after 7.5 mg/kg MTF, orally) after sample pretreatment for DDVP isolation.

TABLE I

INTRA-ASSAY COEFFICIENTS OF VARIATION FOR THE MEASUREMENTS OF MTF AND DDVP IN HUMAN PLASMA

Concentration (μg/ml)	Recovery (%)	C.V. (%)
MTF		
1	32.33	7.76
5	31.87	6.53
10	35.23	3.95
DDVP		
0.15	84.87	8.04
0.75	85.67	1.20
1.50	78.47	1.16

the activity by 2 h and to 0% by 4 h, whereas at 37°C only 7.9% of the original acitivity was remaining by 1 h which declined to 0% by 2 h. DDVP was relatively stable at 4°C with 82.3% of original activity remaining by 4 h but at 22°C and 37°C, 10 and 9.4% was remaining by 4 and 1 h, respectively. The *in vitro* half-life of MTF and DDVP in human plasma was 78.58 and 13.42 h at 4°C, 0.96 and 0.57 h at 22°C and 0.26 and 0.29 h at 37°C, respectively. MTF was stable in doubly distilled water at 4, 22 and at 37°C for at least seven days. DDVP was stable at 4°C upto seven days but degraded to 82.5 and 44.5% of the original amount at 22 and 37°C, respectively, by seven days. Both MTF and DDVP were stable at 37°C in sodium acetate buffer solutions at pH 1, 4 and 7 for at least 1 h but immediately degraded in pH 11 solution.

DISCUSSION

HPLC methods utilising UV detection at 210 nm were developed for the first time in our laboratory for the isolation and quantitation of MTF and DDVP from human plasma. A liquid–liquid extraction method was employed for the isolation of MTF from human plasma to yield consistent recovery for a wide range of drug concentrations [10]. The solubility of MTF at 25°C in water, chloroform and diethyl ether is 154, 750 and 170 mg/ml, respectively, and MTF is very slightly soluble in hexane and pentane [11]. Contaminating peaks from plasma coeluting at the retention time of MTF during chromatography were eliminated using chloroform to extract MTF from plasma. After extraction of MTF into the chloroform layer, it was essential to wash the organic layer with hydrochloric acid followed by drying with a few pieces of drierite ($CaSO_4$) to remove any trace of aqueous contamination which otherwise coeluted at the retention time of MTF. All MTF was recovered when a known amount was added to 3.8 ml of chloroform in the presence or absence of drierite followed by HPLC analysis indicating that there was no loss of MTF due to the drierite. The low recovery of MTF during plasma extraction with chloroform can be attributed to its relatively high solubility in water (15.4 g per 100 ml water, 75 g per 100 ml chloroform) [11].

DDVP is a liquid at room temperature (density, 1.415 g/ml, boiling point at 0.026 bar, 140°C) [11] and therefore any steps to concentrate the samples for its determination by extraction with subsequent evaporation to dryness may result in a loss of DDVP. Adding DDVP (0.3 μg) to hexane (5 ml) followed by evaporation of hexane under a gentle stream of nitrogen, reconstitution in water and injection into the HPLC system did not show any peak at the retention time of DDVP indicating its loss during evaporation. Therefore it is critical that a plasma extraction procedure for DDVP analysis does not involve sample concentration by evaporation. The present method employed protein precipitation by hydrochloric acid followed by filtration and direct injection of the filtrate into the HPLC column. Hydrochloric acid was the most suitable of all precipitating agents tested [6 *M* nitric acid, trifluoroacetic acid or trichloroacetic acid (10%, v/v)].

The only method reported in the literature for the quantitation of MTF and DDVP from human plasma is GC–MS [7]. The authors used liquid–liquid extraction followed by evaporation to dryness for both MTF and DDVP analysis from the same aliquot of a plasma sample which can result in a loss of DDVP during evaporation. The authors have failed to report both recovery and intra-assay coefficients of variation for the GC–MS method.

We have reported elsewhere peak plasma MTF and DDVP levels of 7.2 ± 2.0 μg/ml and 542.3 ± 193.6 ng/ml, respectively, after acute oral administration of 7.5 mg/kg MTF in three AD patients [8,9]. Nordgren *et al.* [12] have reported peak plasma drug concentration of approximately 2.2 and 4.8 μg/ml and 28.33 and 28.6 ng/ml for MTF and DDVP, respectively, measured by GC–MS after a single oral dose of 7.5 mg/kg MTF in young adults. The discrepancy in DDVP levels can be either due to the differences in metabolism rates between young adults and AD patients or attributed to the variation in extraction procedures of the two methods.

Plasma samples containing MTF and DDVP

or solutions of these compounds in doubly distilled water can be stored at −70°C for at least six months. MTF and DDVP degraded under basic conditions and this agrees with the literature report of MTF rearranging to form DDVP at higher pH values [3]. MTF was relatively more stable than DDVP under all conditions tested. The stability of MTF and DDVP in plasma at 4 and 22°C suggests that no temperature-dependent degradation would have occurred during the extraction procedures. The short half-life of MTF (0.26 h) and DDVP (0.29 h) in human plasma at 37°C suggests the rapid enzymatic degradation of these compounds, which should be considered before conducting *in vitro* experiments at 37°C with these compounds.

The HPLC methods we have developed is economical and can be used for the routine monitoring of patient plasma and is sensitive to measure both MTF and DDVP levels at clinically significant concentrations.

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