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Development, validation and application of assays to quantify metrifonate and 2,2-dichlorovinyl dimethylphosphate in human body fluids

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Abstract

Gas chromatographic procedures [GC with electron-capture detection (ECD) and GC–MS] for the quantitative analysis of metrifonate and its active metabolite 2,2-dichlorovinyl dimethylphosphate (DDVP) in human blood and urine were developed, validated, and applied to the analysis of clinical study samples. Analysis of metrifonate involved extraction of acidified blood with ethyl acetate followed by solid-phase clean-up of the organic extract. Acidified urine was extracted with dichloromethane and the residue of evaporated organic phase was reconstituted in toluene. ECD and diethyl analogue of metrifonate internal standard (I.S.) were used for quantitation of metrifonate. The metrifonate lower limit of quantitation (LOQ) was 10.0 μg/l. The DDVP metabolite was chromatographed separately after cyclohexane extraction of acidified blood and urine using d_6-DDVP I.S. and MS detection. The LOQ of DDVP was 1 μg/l. Stability studies have confirmed that the matrix should be acidified prior to storage at −20°C or −80°C to inhibit chemical and enzymatic degradation of the analytes and to avoid overestimation of DDVP concentrations. Metrifonate was found to be stable in acidified human blood after 20 months of storage at −20°C and after 23 months of storage at −80°C. Under these conditions DDVP was found to be stable after 12 months of storage. Both assay procedures were cross-validated by different world-wide laboratories and found to be accurate and robust during analyses of clinical study samples.

Keywords: Metrifonate; 2,2-Dichlorovinyl dimethylphosphate

1. Introduction

Metrifonate [dimethyl-(2,2,2-trichloro-1-hydroxyethyl) phosphonate] (see Fig. 1) is a pro-drug non-enzymatically transformed into the active metabolite 2,2-dichlorovinyl dimethylphosphate (DDVP) which inhibits acetylcholinesterase (AChE) and butyrylcholinesterase [1]. The mechanism of the base-catalysed transformation involves molecular rearrangement starting with the abstraction of the OH-proton of metrifonate [2]. Metrifonate has been used safely by millions of patients for about 30 years for the treatment of schistosomiasis [3], and more recently, it has been shown to be effective in the symptomatic treatment of patients with mild to moderate Alzheim-

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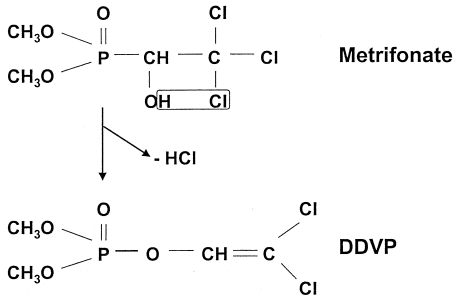

Fig. 1. Chemical structures of analytes.

er's disease (AD) [4]. Inhibition of AChE in the central nervous system of AD patients results in increased levels of the neurotransmitter acetylcholine thereby facilitating treatment of the cholinergic deficit associated with AD [5].

The clinical development of metrifonate for the AD indication required assays to measure drug and active metabolite in biological matrices following administration of metrifonate doses between 50 mg and 80 mg per subject [6]. Previously used gas chromatographic assays for analysis of metrifonate and/or DDVP involved phosphorus detection [7], flame ionisation detection [8], flame photometric detection [9] and MS detection [10,11]. More recently, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) has been successfully employed to determine both analytes in acidified blood of various animal species with limits of quantification (LOQs) of 5.00 μg/l (metrifonate) and 10.0 μg/l (DDVP), respectively [12]. While many published gas chromatography (GC) methods were developed for the relatively higher metrifonate doses used in schistosomiasis, only a few procedures have the necessary sensitivity for analysis of clinical study samples for the AD indication at the reported LOQs of 2 μg/l for DDVP [10] and 2.5 μg/l for metrifonate [13]. Moreover, the problem of chemical instability of the analytes in the matrix [7] has not been properly addressed in all published assays and has more recently become the subject of debate [14,15]. Consequently, our objective is to describe and implement appropriate GC assays for analysis of metrifonate and DDVP in blood and urine, to present validation data and contribute to the debate concerning analyte stability and its effect on measured concentrations of metrifonate and DDVP. Cross-validation between different laboratories and quality control (QC) during routine analysis of clinical studies are further aspects of the present work which become important in order to compare bio-analytical data between different studies and/or sites in the course of clinical drug development.

2. Experimental

2.1. Chemicals

Certified reference compounds of DDVP, *O,O*-[$^2H_6$]dimethyl 2,2-dichlorovinyl phosphate (d_6-DDVP, I.S.), metrifonate and its diethyl analogue (I.S.) were synthesized and released by Bayer AG (Wuppertal, Germany). The purity of the standard compounds was 98.3% (DDVP), >99% (d_6-DDVP), 99.9% (metrifonate), and 99.5% (diethyl metrifonate). The following solvents were used: *n*-hexane analytical-reagent grade (E. Merck, Darmstadt, Germany), ethyl acetate analytical-reagent grade (Baker, Groß-Gerau, Germany), dichloromethane analytical-reagent grade (E. Merck), cyclohexane LiChrosolv (E. Merck), toluene nanograde (Promochem, Wesel, Germany), orthophosphoric acid suprapur (E. Merck). Deionised water was freshly prepared by the Milli-Q system (Millipore Waters, Eschborn, Germany). Solid-phase extraction (SPE) was performed using SPE-Plus-3ML-CN micro-columns (Spec, Irvine, CA, USA). Blank blood and urine were collected from healthy volunteers in the ward of the Institute of Clinical Pharmacology, Bayer AG. Urine was adjusted to pH 2.5 with phosphoric acid prior to use in calibrators or QC samples.

2.2. Stock and working solutions

Stock and working solutions were prepared with certified reference standard compounds in 0.74 *M* phosphoric acid (metrifonate) and acetonitrile (DDVP), respectively.

2.3. Sample preparation

The following descriptions apply to unknown study samples as well as calibrators and QC samples.

2.3.1. Metrifonate in blood

A 100-μl volume of the I.S. working solution (equivalent to 60 ng I.S.) was added to the (200 μl) whole blood–H_3PO_4 mixture (1:1, v/v) in a 10-ml ground-glass tube. Metrifonate and I.S. were extracted from the matrix with 1 ml of ethyl acetate–hexane (70:30, v/v) (10 min at 160 rpm, automatic shaker Certomat R, Braun, Melsungen, Germany). Following centrifugation (15 min at 0°C) the tubes were kept at −20°C for 1 h. The organic phase was then applied to SPE-Plus-3ML-CN micro-columns, that were pre-treated with ethyl acetate–hexane (70:30, v/v, 2×1 ml gravity flow). The eluent was collected in an auto-sampler vial and a 2-μl aliquot was injected onto the GC system.

2.3.2. Metrifonate in urine

A 200-μl amount of urine and 200 μl 0.74 *M* H_3PO_4 were added to the I.S. working solution (equivalent to 150 ng I.S.) in a glass tube. The mixture was vortex-mixed for about 2 s. Metrifonate and I.S. were extracted with 3.5 ml dichloromethane (10 min at 250 rpm, automatic shaker). Following centrifugation (15 min at 0°C) the tubes were kept at −20°C for 1 h. The organic phase was removed with a pipette and evaporated under a gentle stream of nitrogen (37°C, TurboVap, Zymark, Hopkinton, MA, USA). The residue was reconstituted in toluene (200 μl). A 2-μl aliquot of this solution was injected onto the GC system.

2.3.3. DDVP in blood

A 1000-μl volume of whole blood–H_3PO_4 mixture and 250 μl of the I.S. working solution in cyclohexane (equivalent to 20 ng d_6-DDVP) were added to a 1.5-ml micro test tube. The tube was capped, shaken for 40 min on an automatic shaker (speed 400 rpm) and centrifuged for 60 min at approx. 4700 rpm (20°C). The organic phase was transferred into an auto-sampler vial and a 5-μl aliquot was injected onto the GC–MS system.

2.3.4. DDVP in urine

A 250-μl volume of the I.S. working solution was added to a 1.5-ml micro test tube containing 400 μl urine. The mixture was shaken for 30 min on an automatic shaker (speed 400 rpm) and centrifuged for 5 min at approx. 4000 rpm (20°C). The organic phase was transferred into an auto-sampler vial and a 5-μl aliquot was injected onto the GC–MS system.

2.4. Instrumentation and operating conditions

2.4.1. Metrifonate

A HP 5890 II gas chromatograph with split–splitless injector and electron-capture detector was connected to a personal computer with Chemstation software for parameter control and raw data acquisition (Hewlett-Packard, Waldbronn, Germany). A 2-μl volume of the organic extract was injected onto a deactivated single taper liner (No. 5081-3316, Hewlett-Packard) at 200°C with purge-on and purge-off time set to 0.5 min and 22.0 min, respectively. An Ultra 2 column (Hewlett-Packard) of 25 m×0.32 mm, coated with cross-linked 5% phenyl–methylsilicone (0.52 μm film thickness) was operated with helium 5.0 at 1.00 bar. The temperature program for analysis in blood (figures in parentheses pertain to urine) was set as follows: initial temperature: 100 (120)°C, holding time: 1.0 (0.5) min; rate A: 10 (2)°C/min, final temperature: 150 (144)°C, holding time: 1.0 (0.1) min; rate B: 25 (45)°C/min, final temperature: 300 (290)°C, holding time: 5.0 min; rate C: 50°C/min, final temperature: 150 (120)°C, holding time: 0.2 min. The electron-capture detector was operated at 320°C with argon–methane as make-up gas. The retention times were approx. 7.2 min and 8.5 min (pertinent to blood) and approx. 8.5 min and 12.3 min (pertinent to urine) (metrifonate and I.S., respectively). The run time was equal to approx. 22.2 min (blood) and 24.4 min (urine).

2.4.2. DDVP

A HP 5890 II gas chromatograph with split–splitless injector and mass-selective detector MSD 5971 (Hewlett-Packard) was used. The mass-selective detector was operated in the selected ion monitoring (SIM) mode. The following operating conditions were employed: 5 μl of the organic extract was injected at 120°C with the split closed from 0 to 1.0 min. A deactivated insert liner (mixing chamber ass'y No. 19251-60540) with glass-wool was used. The stationary phase [cross-linked methylsilicone No. 19091 A-102, film thickness: 0.33 μm; 25 m× 0.20 mm I.D. (Ultra-1, Hewlett-Packard)] was operated with helium 5.0 as carrier gas at 0.45 bar. The

temperature program was defined as follows: initial temperature: 80°C; initial time: 1 min; rate A: 50°C/min; temperature A: 100°C; hold time A: 0.3 min; rate B: 1.5°C/min; temperature B: 113°C; hold time B: 0 min; rate C: 70°C/min; temperature C: 300°C; hold time C: 2.0 min. For the urine assay, rate B was set to 2°C/min. The ion source was operated in the electron impact mode (70 eV). The transfer-line temperature was 280°C and the detector was set to SIM of m/z 109 (DDVP) and m/z 115 (d_6-DDVP). With a total run time of approx. 15.0 min (blood) and 13.0 min, respectively, (urine) the retention time of DDVP was equal to approx. 11.5 min (blood) and 9.6 min (urine).

2.5. Calibration and quality control

Metrifonate calibrators and QC samples were prepared by adding equal volumes of working solutions (in 0.74 *M* H_3PO_4) to blood. Aliquots of acetonitrile working solutions were spiked into blood–0.74 *M* H_3PO_4 (1:1, v/v) to yield DDVP calibrators and QC samples. Calibrators and QC samples, respectively, were prepared from two independent stock solutions and stored in aliquots at −80°C.

2.6. Assay validation

2.6.1. Accuracy, precision, limit of quantification

The initial validation of each assay comprised the analysis of six replicates of spiked matrix samples per concentration level along with blank matrix samples per day. Usually 4–5 levels covering the working range from the LOQ to the upper limit were investigated and the validation experiment was repeated on three days (i.e., $n_{total}=18$ per concentration level). The LOQ was equal to the lowest concentration that could be quantified with an accuracy of 90–110% and a precision of ≤20%. During routine use QC samples at three concentration levels covering concentrations in study samples were concurrently analyzed to monitor precision and accuracy.

2.6.2. Selectivity

Selectivity was investigated by analysis of extracts of blank matrix and evaluation of interference with similar retention time as compared to analyte and I.S. Additionally, cross-interference was investigated by analyzing DDVP in samples spiked with metrifonate and vice versa.

2.6.3. Recovery

In general, recovery was determined by spiking analyte [to reach concentrations of 40–1000 and 10–500 μg/l (metrifonate; blood and urine) and 24–484 and 2–72 μg/l (DDVP; blood and urine)] and I.S. into blank matrix (a) and the extraction solvent (b), respectively, subjecting the spiked matrix sample to the extraction procedure and calculating the quotient of signal intensities a:b in %. If matrix effects, i.e., different detector responses in matrix extracts as compared to pure solvents were present as in the case of metrifonate, the reference solution (b) was prepared by spiking analyte into an extract of blank matrix.

2.6.4. Stability

Stability of the analytes in the matrix was assessed by means of spiked matrix samples and actual study samples, stored at different temperatures [freezer (−80°C, −20°C and 4°C) and ambient; storage at −80°C was used for thaw–freeze cycles]. The number of replicates per experiment is indicated in Table 1. Stability was investigated in each matrix and solvent relevant to the assay, i.e., blood, blood–phosphoric acid, acidified urine, extraction solvent, reconstitution solvent (auto-sampler stability), and solvent for stock and working solutions. Stability in solvents was investigated at 4°C and ambient temperature.

2.6.5. Cross-validation

Cross-validations between different laboratories world-wide were performed by spiking the analyte into blank matrix at five to eight different concentrations (spread across working range) in laboratory B and shipping six replicates of each concentration along with six blank matrix samples in random order to laboratory A (the latter being the laboratory to adapt the assay already validated in laboratory B). Laboratory A remained blinded and accuracy and precision were evaluated by laboratory B.

Table 1
Stability of DDVP and metrifonate in blood–phosphoric acid mixture

Storage temperature (°C)	Initial concentration (μg/l)	*n*	Sample type[1]	Time of storage (months)	Concentration after storage (% of initial value; mean±SD)
DDVP					
−80	24.2–484	9	a	0.8	104.1±8.6
	4.5–144	18	a	15	87.4±3.2
	30.0–43.2	20	b	22	83.6±5.1
−20	10.3	18	a	12.5	108.7±3.6
	82.0	18	a	12.5	109.1±2.0
Metrifonate					
−80	3050–5870	18	b	11	95.0±10.7
	66–393	10	b	23	109.4±7.4
−20	500	15	a	3.6	104.3±2.4
	2500	15	a	3.6	98.9±6.9
	624–2060	12	b	20.5	102.6±7.0

[1] a: Calibrator or QC sample spiked with metrifonate; b: real study sample containing metrifonate and DDVP.

2.7. Evaluation of raw data

Chromatographic raw data were evaluated using the personal computer software CONCALC (Institute of Clinical Pharmacology, Bayer AG). Using internal standardization, calibration curves were constructed by plotting relative peak-height of analyte: I.S. versus concentration and fitting linear or non-linear equations to the data points. The type of equation was selected in order to minimize relative residuals. $1/y^2$-weighting was used for linear regression curves. Calibrators and QC samples were randomly distributed among unknown samples in sequences. Acceptance criteria for sequences were: relative residuals (i) within ±20% in calibrators, (ii) within ±20% in four out of six QC samples, and (iii) within ±20% in one QC sample of each concentration level.

2.8. Clinical–pharmacological study

An 80-mg amount of metrifonate was administered as tablet to 12 healthy male Caucasian volunteers at 8:00 a.m. after overnight fasting as part of a three-way cross-over study. The study was approved by a local ethics committee and conducted according to Good Clinical Practice Guidelines, and the 1975 Declaration of Helsinki and its revisions. The subjects gave their written informed consent to participate. Blood samples (2×1.5 ml) for assay of metrifonate and DDVP were collected before administration and 10, 20, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8 and 12 h post-administration. Blood was acidified with an equal volume of 0.74 *M* H_3PO_4 within 2 min after sampling [7] and stored at −80°C until analysis. Urine, collected in the intervals 0–12 h and 12–24 h post-administration, was acidified (pH 2–3) with H_3PO_4 immediately after collection and stored at −40°C until the time of analysis.

3. Results

3.1. Conversion of metrifonate into DDVP ex vivo and general aspects of assay development

In view of the instability of the analytes in blood, experiments were performed to mimic the conditions of blood sample collection: a concentration of metrifonate equivalent to C_{max} (80 mg dose) was spiked into blood at an initial temperature of 37°C and the time-course of DDVP formation was measured (Fig. 2). After 60 min the concentration of DDVP had increased by six-fold compared to the initial value. DDVP was not detectable if metrifonate was spiked

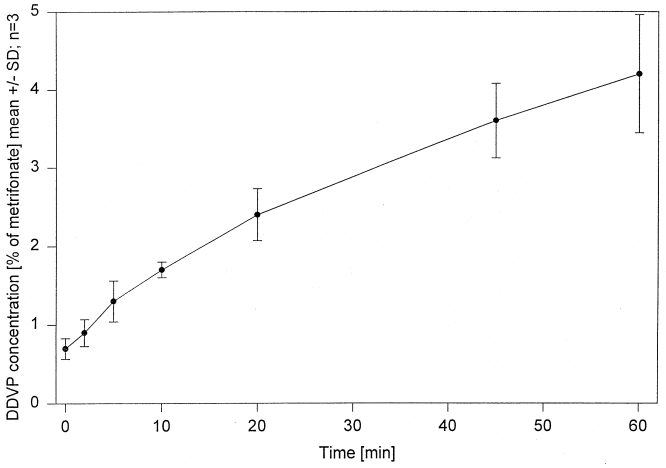

Fig. 2. Time-course of formation of DDVP in blood spiked with metrifonate at 781 μg/l (initial temperature of 37°C equilibrating with ambient temperature in the course of the experiment; $n=3$ per sampling time, $n_{total}=21$).

into blood which was subsequently mixed with an equal volume of 0.74 *M* phosphoric acid [7]. On the other hand, DDVP itself was not stable in blood as well. Its degradation (Fig. 3) appeared to be biphasic and concentration-dependent with a first more rapid decay ($t_{1/2}$~13 min) and a second exponential phase with a half-life of ~30–36 min. Again, the acidification of blood significantly enhanced the stability of DDVP. Based on these results the assays were developed for the matrix blood: phosphoric acid and the sample-handling proposed by Villén et al. [7] was implemented in clinical studies with metrifonate.

For determination of metrifonate capillary GC on cross-linked 5% phenyl methylsilicone was employed. Using a capillary with a film-thickness of 0.52 μm enhanced the robustness of the assay. ECD was used as the LOQ attained with mass-selective detection (40.0 μg/l) was inferior to that of the ECD

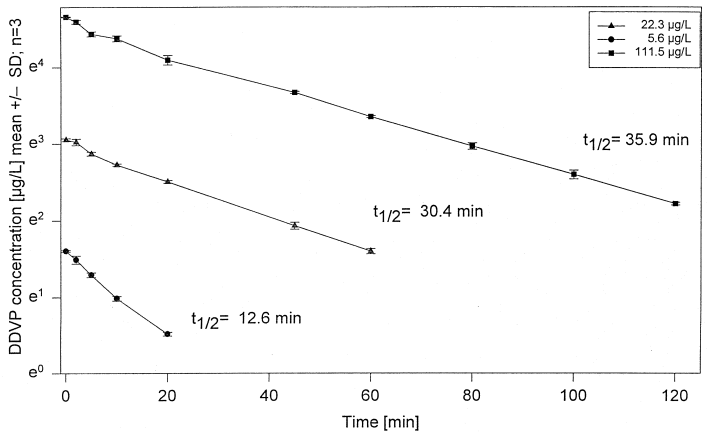

Fig. 3. Time-course of disappearance of DDVP in blood spiked with DDVP at different concentrations (initial temperature of 37°C equilibrating with ambient temperature in the course of the experiment; $n=3$ per sampling time, $n_{total}=71$).

method (see below). The diethyl analogue of metrifonate was chosen as I.S. Liquid–liquid extraction of blood–phosphoric acid with 70% ethyl acetate in hexane followed by filtration of the organic phase through SPE-CN micro-columns was found to be superior to other investigated sample preparation techniques in terms of robustness, selectivity and absence of matrix effects, allowing an LOQ of 10.0 μg/l.

Mass-selective detection in the SIM mode was employed for analysis of DDVP. The selected ion *m/z* 109 [$(CH_3O)_2P^+=O$] represents the base-peak in the electron-impact mass spectrum of DDVP [16]. In conjunction with cyclohexane liquid–liquid extraction and capillary GC on cross-linked methylsilicone, GC–mass selective detection facilitated the selective and sensitive quantification of DDVP. Moreover, mass-selective detection also allows the use of an isotope-labeled analogue of the analyte (d_6-DDVP) as I.S. which was detected at *m/z* 115 [$(C^2H_3O)_2P^+=O$]. ECD, investigated as alternative detection method, turned out to be slightly less sensitive (LOQ of 2.50 μg/l) and – more importantly – less selective and more prone to interference from compounds emanating from plastic in sample tubes. The volatility of DDVP which is a liquid at ambient temperature was an important limitation in the development of the assay as evaporation of extracts had to be avoided.

The assay described for DDVP in blood was also applicable to urine. Validation results are presented below. In the case of metrifonate, however, the extraction from urine could be simplified as further clean-up of the organic extract was not required in the absence of matrix effects. Dichloromethane was used as extraction solvent.

3.2. Metrifonate

3.2.1. Stability in solvents

Metrifonate was stable in 0.74 *M* H_3PO_4 (the solvent for stock/working solutions) for at least 7 months (4°C). There was no degradation in dichloromethane (the extraction solvent for urine) up to 3.5 h and metrifonate and I.S. were stable in toluene (injection solvent, ambient temperature) for at least 9 days.

3.2.2. Validation of method for determination of metrifonate concentrations in acidified blood

In the employed chromatographic system there was no significant interference at the retention time of metrifonate or I.S. in matrix-blanks (Fig. 4). The metabolite DDVP also did not interfere with analyte or I.S.

Relative residuals in calibration curves were minimized by fitting the relative peak-height vs. concentration data to the equation $y=a+bx^c$ using iterative log/log regression. The recovery of the combined liquid–liquid–solid-phase extraction procedure was 63.2±6.8% (mean±SD; metrifonate 40–1000 μg/l, $n=18$) and 83.7±8.5% (I.S. working concentration, $n=18$). Results of a 3-day validation experiment are given in Table 2. 10.0 μg/l was defined as LOQ of the assay. A cross-validation carried out between laboratories in the USA (GC–ECD) and Japan (GC–mass-selective detection) was completed with accuracy and precision in the working range of 103–110% and ≤6.2%, respectively. The assay specifications during analysis of study samples were assessed by means of concurrent QC samples: in a study with $n=468$ unknown samples inter-day accuracy and precision were 93.6–100.9% and 6.4–8.5%, respectively (40.0–1600 μg/l, $n=20$ per concentration) which is comparable to the validation results (Table 2). Stability experiments showed that there was no relevant decrease in metrifonate concentration in blood–H_3PO_4 after three freeze–thaw cycles with 91±7.5% of the initial concentration observed after the third cycle ($n=27$, 75.0–2500 μg/l). Metrifonate was stable in acidified blood for about 1 month (ambient temperature) and for up to 220 days (4°C), respectively. Metrifonate was stable at both −20°C and −80°C for 20 months and 23 months, respectively (see Table 1).

3.2.3. Validation of method for determination of metrifonate concentrations in acidified urine

The method was selective with blank urine containing a small and constant interfering peak equal to about 15% of the peak-height of metrifonate at its LOQ of 10.0 μg/l. Complete validation data is given in Table 2. Inter-day precision of the assay during routine use was ≤6.7% (10–1625 μg/l). Recoveries of analyte and I.S. from urine using dichloromethane

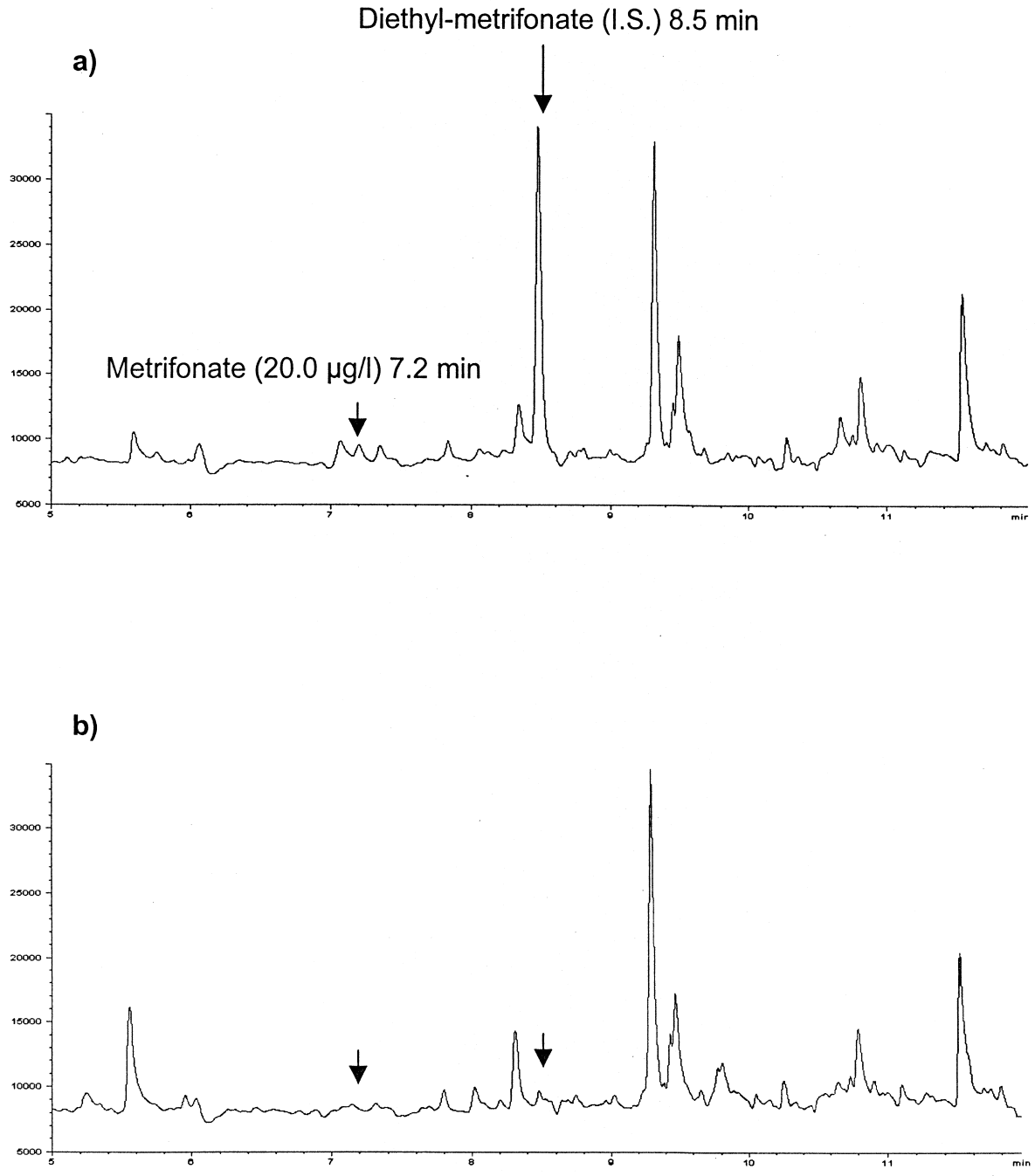

Fig. 4. Chromatograms of blood spiked with I.S. and metrifonate at 20.0 μg/l (a) and blank blood (b).

liquid–liquid extraction were found to be 53.7±8.6% and 43.8±4.7%, respectively (10.0–500 μg/l, $n=8$). Metrifonate was stable (i.e., concentrations were within 90–110% of initial values) in urine at pH 2–3 for at least 70 days at −20°C and −80°C and for at least 7 days at 4°C, 23°C and 37°C. Two sequential

Table 2
Accuracy and precision of the assays to determine metrifonate and DDVP in urine and blood (n=18 per concentration)

	DDVP in urine				DDVP in blood					Metrifonate in urine					Metrifonate in blood				
	Nominal concentration (μg/l)																		
	2.44 (LOQ)[a]	4.88	30.7	65.1	1.13 (LOQ)[b]	5.32	42.6	213	426	10.0 (LOQ)	20.0	25.0	150	350	10.0 (LOQ)	20.0	40.0	600	1600
Precision inter-day (%)	13.1	1.8	2.2	4.1	3.7	5.2	3.3	3.8	3.3	12.7	12.7	8.1	13.0	6.0	7.9	9.9	10.9	5.4	5.4
Accuracy inter-day (%)	90.2	93.8	94.0	92.8	96.4	109.6	112.1	109.0	107.7	96.7	96.6	102.4	103.9	101.4	100.1	93.8	97.6	97.2	97.1

[a] LOQ of 1.18 μg/l was later revalidated (accuracy 98.5%, precision 3.9%; n=6).
[b] n=15.

freeze–thaw cycles also had no effect on the measured concentrations.

3.3. DDVP

3.3.1. Stability in solvents

DDVP was stable in acetonitrile (the solvent for stock and working solutions; 4°C and ambient temperature) and cyclohexane (the extraction/injection solvent; ambient temperature) for at least 11 weeks.

3.3.2. Validation of method for determination of DDVP concentrations in acidified blood

DDVP was quantified in the SIM mode employing the signal m/z 109. A matrix-derived signal equivalent in height to a DDVP concentration of approximately 2 μg/l was present which could not be completely baseline-separated (Fig. 5). DDVP could be quantified down to a concentration of 1 μg/l (i.e., the LOQ of the assay).

Analysis of the signal at m/z 109 after injection of the I.S. reference compound showed that this interference is not due to a non-labeled DDVP impurity in d_6-DDVP. In blood–H_3PO_4 spiked with metrifonate (1000 μg/l, approximately equivalent to C_{max} values in clinical studies) and subjected to the assay for DDVP, DDVP was either not detectable or equivalent to 0.1% of the initial metrifonate value (5390 μg/l metrifonate, a concentration approx. fivefold higher than clinically relevant). Calibration curves were described by the equation $y=a+bx^c$ in plots of relative peak height (DDVP:d_6-DDVP) vs. concentration. Complete validation data is given in Table 2. Cross-validations in the clinically relevant concentration range were performed between two European laboratories as well as sites in Japan (GC–mass-selective detection assay) and the USA (alternative GC–ECD assay). Accuracy between the involved laboratories fell within the acceptance range of 90–110%, and inter-laboratory precision was ≤6.7%. The assay specifications during routine application based on concurrent analysis of QC samples were 98.5–101.9% (inter-day accuracy) and 5.0–7.9% (precision), respectively (3.68–19.6 μg/l; n=20 in a study with n=468 unknown samples) confirming the validation results (Table 2). Recovery of DDVP and d_6-DDVP after cyclohexane extraction was 41.1±3.1% and 37.4±3.3%, respectively. Four repeated freeze–thaw cycles had no influence on the measured DDVP concentrations in blood–H_3PO_4 spiked with DDVP. Two freeze–thaw cycles did not increase DDVP concentrations in study samples containing metrifonate in 100-fold excess, indicating that the metabolite is not formed from parent drug during freezing–thawing (97.7±8.7% of initial DDVP concentration (1–26 μg/l) were found upon re-assay, n=35). Upon storage in the blood–H_3PO_4 matrix at ambient temperature and 4°C, DDVP concentrations remained within 90 to 110% of the initial value for 2 days and 14 days, respectively, while there was clear evidence for decomposition upon long-term storage with an estimated half-life of 15 days (ambient) and 103 days (4°C). At temperatures employed for long-term storage of study samples, DDVP appeared to be stable for 1 year, while samples stored for 15 or 22 months showed a trend towards decomposition of the analyte (Table 1).

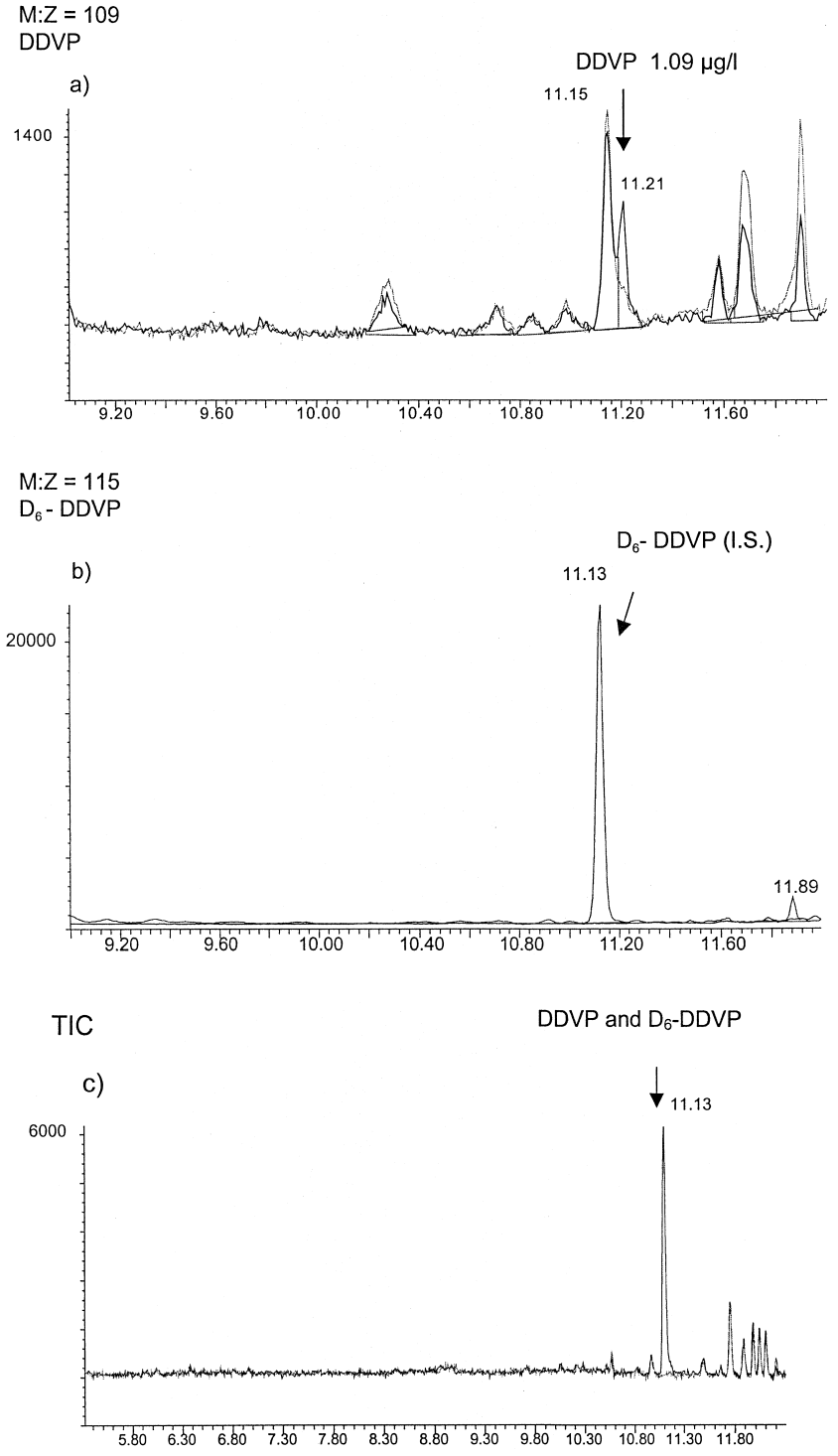

Fig. 5. Chromatograms of blood spiked with DDVP at 1.09 µg/l and I.S., and blank blood. Trace of DDVP (a), trace of I.S. (b) and total ion chromatogram (TIC, c); dotted lines represent blanks.

3.3.3. Validation of method for determination of DDVP concentrations in acidified urine

In the absence of relevant matrix-interference DDVP was quantified in urine with a LOQ of 2.44 and 1.18 μg/l (revalidation, $n=6$), respectively. Validation data are given in Table 2. Inter-day precision of the assay during routine use was ≤3.9% (1.18–81.8 μg/l). Recovery of DDVP was 52±2.6% in the range of concentrations from 2 to 72 μg/l. Relevant degradation in urine (pH 2–3) was observed after 4 h (37°C), 1 day (ambient temperature) and 7 days (4°C), respectively. Under the conditions of sample storage (−20°C) DDVP was stable in urine for at least 70 days.

4. Application of the described assays

Maximum metrifonate and DDVP blood concentrations of 739 μg/l and 6.33 μg/l were observed 20 min and 30 min, respectively, after administration of 80 mg metrifonate and the elimination profiles of both analytes were in parallel (Fig. 6). Concentrations above LOQ were measurable up to 6–8 h and DDVP concentrations were equal to 0.9–1.9% of metrifonate values. 1.42% (metrifonate) and 0.01% (DDVP) of the dose was recovered in urine within 24 h post-administration.

5. Discussion and conclusion

Separate assays for metrifonate and DDVP were developed although simultaneous quantification in the same chromatographic system has been demonstrated [17]. This strategy was chosen (i) in order to avoid overestimation of DDVP due to conversion of metrifonate into DDVP in the GC apparatus, and (ii) to facilitate evaporation/reconstitution steps for metrifonate which were to be avoided for DDVP. The DDVP assay tended to be more precise compared to metrifonate, which may be due to the lack of discrimination between the isotope-labeled I.S. and analyte in the case of DDVP.

Recoveries of the present assays are moderate [41% (DDVP) and 63% (metrifonate)], however, experiments with other solvents suggested that selectivity has to be sacrificed in order to increase recovery. The assays can be applied to clinical studies as indicated by a C_{max}/LOQ (blood) ratio of approximately 70 (metrifonate) and 12 (DDVP), respectively, following a 50 mg metrifonate dose [18]. Sensitivity was also adequate to quantify both

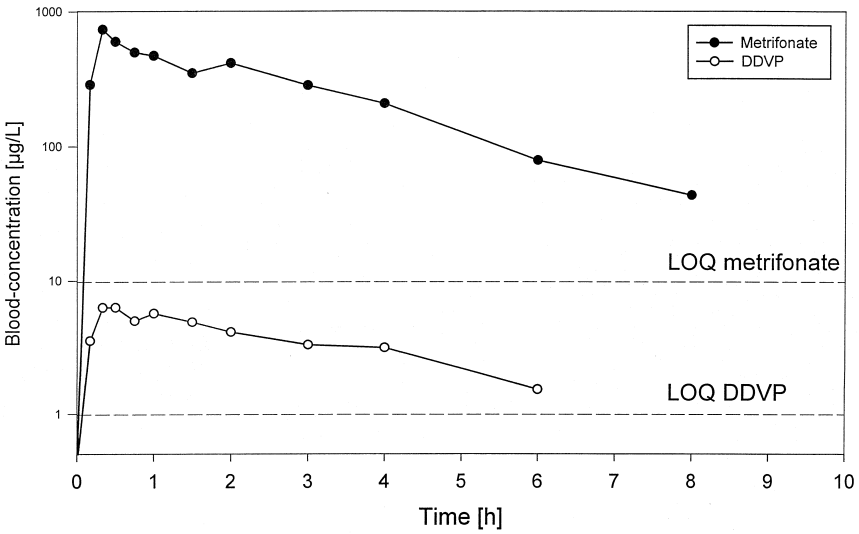

Fig. 6. Blood concentration vs. time profile of metrifonate and DDVP in healthy male volunteer following a single administration of a metrifonate 80 mg tablet.

analytes in urine although their renal excretion is minimal. The assays complied with the requirements of regulatory guidelines [19,20] and demonstrated their robustness by way of successful inter-laboratory cross-validations and consistent accuracy and precision during routine application. Based on these quality control measures concentration data between different laboratories, studies and sites can be compared and meta-analyses and population–pharmacokinetic evaluations can be conducted.

The present investigations were carried out with blood: phosphoric acid as there is significant ex vivo formation of DDVP from metrifonate during plasma preparation. With metrifonate present in blood at concentrations approx. 100-fold higher than DDVP the net result is an overestimation of the in vivo concentration of DDVP [7]. DDVP itself is also unstable in blood resulting from esterase activity, a process which is also effectively inhibited by phosphoric acid. The decay profiles of DDVP in blood appeared to be biphasic and $t_{1/2}$ values were concentration-dependent, indicating that the observed decrease in concentration is the sum of two processes i.e., (i) covalent binding of DDVP to cholinesterases and (ii) esterase-catalysed hydrolysis.

More recently, the importance of stabilizing the analytes in the matrix was questioned [14,15] following the publication of pharmacokinetic studies with collection of plasma samples in the absence of pH-adjustment [21,22]. In plasma without phosphoric acid the mean concentration ratio (DDVP:metrifonate) was increased to 7.5% [21,22] compared to 1–2% [7,11,23,24] in studies using blood–phosphoric acid. It was speculated that differences in metabolic rates between young and old subjects may explain this finding [15], as the elevated ratio was observed in elderly patients (58–68 years [22]). Recent studies in elderly subjects, with collection of blood: phosphoric acid samples, indicate ratios (DDVP:metrifonate) of 1–2% (46–73 years [18]), 1.4–3.0% (45–75 years [25,26]) and 2.8–3.9% (55–85 years [27]), respectively, i.e., ratios of up to 11% [22] could not be confirmed. Moreover, a statistically significant effect of age on the pharmacokinetics of DDVP was absent in a meta-analysis of various studies (data on file, Bayer AG) contradicting the hypothetical [15] increase in DDVP concentrations with age. We conclude from the above that the use of phosphoric acid–blood (or other appropriate and validated stabilisation measures) in pharmacokinetic studies with metrifonate and DDVP is imperative in order to avoid erroneous DDVP concentration data.

Both metrifonate and DDVP demonstrated good long-term stability after adding phosphoric acid to the matrices. The observed storage stability of metrifonate for 20 to 23 months is slightly contradictory to earlier reports of a decrease in concentration by 40% after 20 months while the relatively lower stability of DDVP confirms previous results [7].

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