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Internal standard high-performance liquid chromatography method for the determination of obidoxime in urine of organophosphate-poisoned patients

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Abstract

Obidoxime is an antidote approved for reactivation of inhibited acetylcholinesterase in organophosphate poisoning. HPLC methods were described for its determination in blood or aqueous solutions but not for the determination in urine. Since data for renal obidoxime excretion ranged from 2.2 to 84% of administered dose in healthy volunteers depending on the route of administration and little is known about pharmacokinetics of obidoxime in severely intoxicated patients we developed an internal standard (HI 6) reversed-phase HPLC method for determining obidoxime in urine. The mobile phase consisted of methanol, the counter ion 1-heptane sulfonic acid and tetrabutylammonium phosphate, the stationary phase involved a 5 μm reversed-phase column (125×4 mm). Obidoxime was detected spectrophotometrically at 288 nm. The limit of quantification (LOQ) was 1 μ*M*, the limit of detection (LOD) 0.5 μ*M*. Linear calibration curves were obtained in a concentration range from 1 to 1000 μ*M*. Intra- and inter-day precision C.V.s were below 4%. Accuracy was 95.9% in the LOQ range. Using this method, we were able to quantify obidoxime in urine of an organophosphate poisoned patient. Based on this data we calculated that 58% of the administered dose was excreted in the urine.

Keywords: Obidoxime; Organophosphate

1. Introduction

About 200 cases of organophosphate (OP) poisoning, mostly due to suicidal attempts, occur in Germany per year [1]. Atropine is suitable to counteract the muscarinic effects of the ensuing cholinergic crisis [2]. However, it is ineffective at nicotinic receptor sites and cannot restore the function of the respiratory muscles. To improve neuromuscular function and to diminish respiratory insufficiency, reactivators of inhibited acetylcholinesterase (AChE) have been developed [2]. Obidoxime [*N,N′*-oxydimethylene bis(pyridinium-4-aldoxime) dichloride], a fast reactivating oxime [3], is approved in Germany and several other European countries. However, little is known about the pharmacokinetics of obidoxime in severely poisoned patients. Thiermann et al. have shown that pharmacokinetics of obidoxime may change during treatment in patients suffering from organophosphate poisoning [4]. This ob-

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servation was based on determination of obidoxime in plasma, using the high-performance liquid chromatography (HPLC) method described by Spöhrer and Eyer [3]. To obtain more complete data we felt it to be necessary to measure obidoxime concentration in urine samples. To our knowledge there is no study published using HPLC for quantification of obidoxime in urine. Existing HPLC methods for obidoxime quantification were used either for separation of isomers of obidoxime [3], determination of obidoxime in automatic injection devices [5] or measurement of obidoxime concentration in blood [6]. These studies described external standard methods for quantification of obidoxime. However, only Benshop et al. mentioned the possibility of using HI 6 as an internal standard for determination of obidoxime [6]. Since this method showed peak tailing and interference of HI 6, syn–anti and syn–syn obidoxime in chromatography, we developed our own internal standard high-performance liquid chromatography method for the determination of obidoxime in urine of patients suffering from organophosphate poisoning. Using our method, we were able to calculate obidoxime excretion of a 50 year old Caucasian female who tried to commit suicide by injecting 2 ml of parathion into her abdominal wall.

2. Experimental

2.1. Reagents and materials

Obidoxime dichloride and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany) and methanol from Fisons (Loughborough, UK). HI 6 (Fig. 1) was a gift from Dr. Lundy, Canada. 1-heptane sulfonic acid and tetrabutylammonium phosphate were obtained from Waters-Millipore (Eschborn, Germany).

2.2. Sample preparation

One hundred μl of internal standard (HI 6, 10 m*M*) were added to 900 μl of the urine sample and mixed for 1 min. This solution was diluted with distilled water 1:10. One ml of the diluted sample was deproteinated with 300 μl of 1 *M* trichloroacetic acid and centrifuged for 2 mm at 10 000 *g* (Hettich

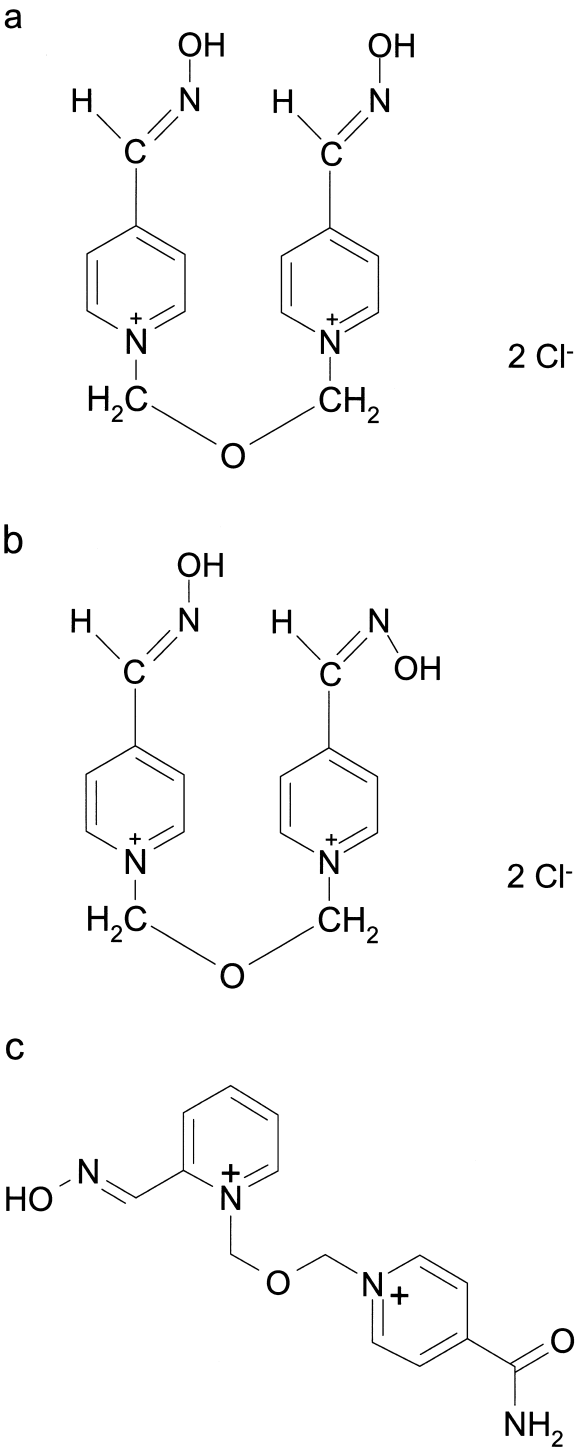

Fig. 1. Chemical structure of a. syn–syn obidoxime, b. syn–anti obidoxime and c. HI 6.

Mikro 12-24 S002-K, Tuttlingen, Germany). Six hundred μl of the supernatant were added to 200 μl 1-heptane sulfonic acid/NH_3 (25/75, v/v; pH 6.0) and 50 μl of this solution were injected into the loop.

2.3. HPLC-analysis

The chromatographic system consisted of an M 480 S solvent delivery system, an Erma ERC 3522 degasser, a GINA 160 sample injector, an STH 585 column thermostat, a 320 S UV detector and a Magix 486 DX 33 computing integrator, all from Gynkotek (Munich, Germany). The stationary phase was a LiChrospher 60 RP select B reversed-phase column (Merck), 125×4 mm I.D., 5 μm particle size.

The mobile phase consisted of methanol-1-heptane–sulfonic acid–tetrabutylammonium phosphate (120:40:5 ml, diluted to 1000 ml with distilled water; pH 5.7) and was delivered at a flow rate of 1.2 ml/mm. The detection wavelength was set to 288 nm.

3. Results

3.1. Linearity of calibration curves

Twenty calibration curves were determined on twenty different days with obidoxime concentration ranging from 1 to 1000 μ*M* (correlation coefficient= 0.9991).

3.2. Stability of frozen obidoxime samples

Obidoxime samples in urine (10, 100, 250, 750 μ*M*; $n=3$, respectively) were stable for at least 2 months when stored at −60°C.

3.3. Precision

Intra-day precision was determined by repeating the measurement of urine samples ten times with five different concentrations (10, 100, 250, 500, 750 μ*M*) on the same day. Inter-day precision was determined by measuring the means of three replicates of urine samples (10, 100, 250, 500, 750 μ*M*) on three different days.

The intra-day precision coefficient of variation (C.V.) was 3.3% (9.9±0.3 μ*M*, mean value±standard deviation), 1.3% (101.6±1.3 μ*M*), 0.5% (251.6±1.1 μ*M*), 0.2% (501.9±1.1 μ*M*) and 1.0% (746.4±7.5 μ*M*), respectively. Inter-day precision C.V. was 3.5% (10.0±0.4 μ*M*), 4.1% (102.1±4.1 μ*M*), 0.6% (250.4±1.5 μ*M*), 0.4% (500.8±1.8 μ*M*) and 0.1% (751.2±1.0 μ*M*), respectively.

3.4. Accuracy

Obidoxime in urine samples (10, 100, 750 μ*M*) was quantified once daily on ten different days. Accuracy was 99.9% (10 μ*M*), 98.1% (100 μ*M*) and 99.2% (750 μ*M*), respectively.

3.5. Limits

The limit of quantification was 1 μ*M* (accuracy 95.9%, $n=5$), the limit of detection 0.5 μ*M*. The limit of detection was defined as a response peak 3× the background noise.

3.6. Recovery

Absolute recovery was the ratio achieved from obidoxime surface area after urine sample preparation and surface area of pure obidoxime concentrations (10, 100, 250, 500, 750 μ*M*, $n=5$, respectively) [7]. It was estimated to be 54% (10 μ*M*), 56% (100 μ*M*), 58% (250 μ*M*), 62% (500 μ*M*) and 63% (750 μ*M*), respectively.

Relative recovery was achieved from obidoxime surface area after urine sample preparation compared to obidoxime surface area achieved after sample preparation from an indifferent matrix, distilled water (10, 100, 250, 500, 750 μ*M*, $n=5$, respectively) [7]. It was determined to be 91% (10 μ*M*), 94% (100 μ*M*), 98% (250 μ*M*), 99% (500 μ*M*) and 99% (750 μ*M*), respectively.

3.7. Chromatography

Urine samples from six volunteers (four male, two female) were used as blanks and for calibration curves [8]. There was no interference with the obidoxime peak. Fig. 2 shows a chromatogram from an organophosphate intoxicated patient with an

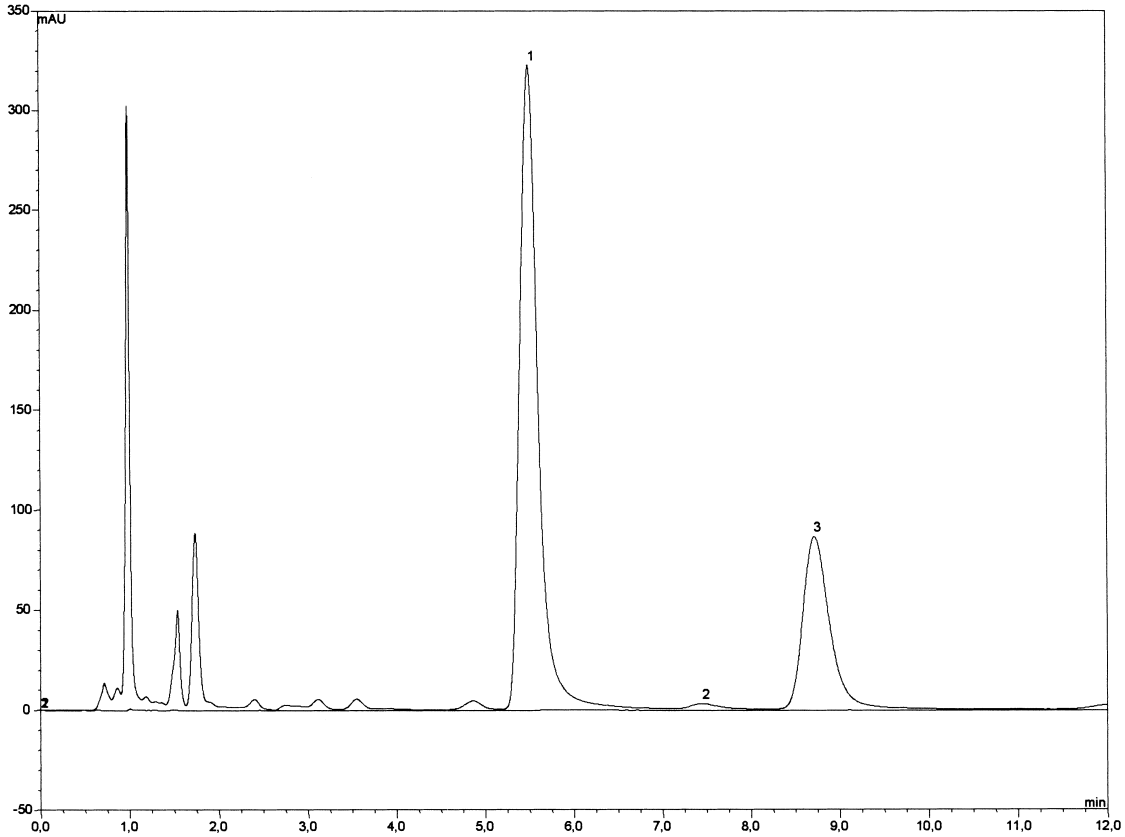

Fig. 2. Chromatogram of obidoxime with underlying blank. The urine sample was obtained from an organophosphate intoxicated patient (concentration=662.1 μ*M*). For sample preparation and chromatographic conditions see Experimental. Peak 1: HI 6 (internal standard), peak 2: syn–anti obidoxime, peak 3: syn–syn obidoxime.

underlying urine blank. Even the separation of syn–anti and syn–syn isomers was possible.

3.8. Case report

A 50 year old Caucasian female tried to commit suicide by injecting 2 ml of parathion into her abdominal wall. She was admitted to ICU 2 days later and was mechanically ventilated for ten days. An obidoxime bolus of 250 mg was applied followed by continuous daily infusion of 750 mg as recently recommended by several authors [4,9,10]. Obidoxime administration was stopped after 7 days. After 1 month, she could be discharged from hospital.

The total dose of administered obidoxime was 5179.7 mg within 7 days. 2992.8 mg were excreted in the urine, corresponding to 57.8% of the applied dose. Obidoxime concentrations in urine samples ranged from 329 to 1445 μ*M* (Table 1).

4. Discussion

In this study we used an internal standard HPLC method for quantification of obidoxime in the urine of an organophosphate intoxicated patient. The sample preparation used resulted in minimisation of interference of urine constituents with chromatographic analysis. Compared to preparation of plasma samples, urine samples were diluted to reduce the loading of the chromatographic system since higher obidoxime concentrations were found in urine compared to plasma. We used the oxime HI 6 as internal standard to achieve high accuracy [11]. HI 6 exhibits

Table 1
Concentrations of obidoxime in urine during obidoxime administration to an organophosphate intoxicated patient

Time after start of obidoxime infusion (h)	Concentration in urine sample (μM)	Excreted dose of obidoxime (mg)	Administered dose of obidoxime (mg)
0–30:45	1445.4	747.7	1195.3
30:45–54:45	662.1	285.4	750.0
54:45–78:45	328.9	419.4	750.0
78:45–102:45	497.8	608.0	750.0
102:45–126:45	329.4	401.1	750.0
126:45–150:45	518.1	390.8	750.0
150:45–174:15	575.3	140.5	234.4

similar chemical properties compared to obidoxime and did not interfere with obidoxime regarding retention time. Linear calibration curves were obtained in a concentration range from 1 to 1000 μM. The majority of obidoxime urine concentrations varied between 300 and 700 μM (Table 1). However, the lower limit of detection of the method would be 0.5 μM, provided an appropriate calibration set was employed, which is in agreement with the method published by Bentur et al. who calculated a detection limit of 1 μM for obidoxime [6]. As shown in Fig. 2, even the separation of syn–anti and syn–syn isomers was possible [3].

Little is known about the pharmacokinetics of obidoxime in organophosphate poisoned patients. Data for renal excretion of the drug obtained from healthy volunteers varied between 2.2% after oral [12], 68% after intravenous [13] and 84% after intramuscular administration [14]. Data taken from a case report published by Bentur et al. [15] showed that 80% of the administered obidoxime was found in urine after 5 h. In our patient, 58% of the administered obidoxime dose was excreted in the urine.

Recent theoretical and experimental work considering pharmacodynamic and pharmacokinetic properties of obidoxime with respect to toxicokinetics of various organophosphates led to the recommendation to use a loading dose (250 mg) followed by continuous infusion at low daily doses (750 mg) instead of single dose injections [9,10]. Since there are several factors that influence pharmacokinetics of obidoxime in the severely intoxicated patient, e.g. renal failure and artificial ventilation [16,17], there is a need to obtain more information about obidoxime pharmacokinetics, especially obidoxime excretion when using continuous obidoxime administration. The internal standard HPLC method described in this study is a reliable method with high precision and accuracy which can be used as a routine method for obidoxime quantification in urine.

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