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HPLC ANALYSIS OF MICROSOMAL METABOLISM OF K-48

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SUMMARY

K-27 and K-48, bisquaternary pyridinium aldoximes differing only in the number of methyl units in the alkyl bridges between the two quaternary nitrogen atoms, are promising candidates for use as potent and effective antidotes to organophosphate intoxication. K-27 and K-48 were treated with rat hepatic microsomal preparations from healthy (control) and diabetic animals. Microsomal metabolism was measured at different times up to 45 min. This treatment resulted in a decrease of approximately 30% in the concentration of K-48 whereas that of K-27 was unchanged. There was no significant difference between the effects of microsomal preparations from control and diabetic rat liver.

K-27 and K-48 in a variety of media were analysed by reversed-phase HPLC with both ultraviolet and electrochemical detection. The method was validated by the usual procedures.

INTRODUCTION

Organophosphate intoxication occurs as a result of contact with insecticides and pesticides. The World Health Organization reports about one million cases of organophosphate intoxication per year. Warfare nerve agents (e.g. sarin and tabun) have also caused the death of many people – known cases are the sarin attack on the Tokyo Metro and use by Saddam Hussein in Iraq. Treatment for organophosphate intoxication includes use of an acetylcholine antagonist (atropine) and regeneration of the acetylcholine esterase enzyme. The latter can be accomplished by use of pyridinium aldoximes. The therapeutic efficacy of recently used aldoximes is, however, far from that expected.

Two major types of pyridinium aldoxime have been synthesized – monopyridinium compounds, for example pralidoxime, and bis-pyridinium compounds, for example obidoxime (for a review, see Petroianu and Kalász [1]). The chemical mechanism is elimination of organophosphate bound to the hydroxyl group of the serine part of acetylcholine esterase [2–5].

Kuca et al. have synthesized a wide variety of bis-pyridinium aldoximes [6–15] with symmetrical and asymmetrical structures and a variety of alkyl bridges between the pyridinium nitrogen atoms. These pyridinium aldoximes were given the letter K and a serial number. Their choline esterase-regenerating activity was screened by use of in-vitro and in-vivo methods, partly by Kuca's group. Petroianu and coworkers have elucidated the effects of the most promising choline esterase-reactivating K-compounds in vitro [16–18] and the survival of rats exposed to organophosphates [19, 20]. Penetration of bis-pyridinium aldoximes through the blood–brain barrier has also been measured experimentally [18,21–23]. Although all of these pyridinium aldoximes are hydrophilic [24,25], a proportion (3 to 8%) can cross the blood–brain barrier [21].

The first attempts at reliable determination of pralidoxime (2-PAM) were by paper chromatography [26,27], but multiple spots were observed even when a single component was chromatographed [27]. Utley [28] and Gibbon and Way [29] determined pralidoxime concentrations in biological samples by column chromatography as early as forty years ago. Grasshoff et al. [30] studied urinary elimination of obidoxime, and Kiderlen et al. [5] found that diethylphosphorylobidoxime is excreted. Moore et al. [31] followed the pharmacokinetics of HI-6 and 2-PAM (two commercially available pyridinium aldoximes), and Lundy et al. [32] and Paddle and Dowling [33] studied the pharmacokinetics in intoxicated subjects.

Progress in separation techniques has enabled the use of ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC) for determination of pralidoxime [21,22,27,34], obidoxime [5,33,35–37], HI-6 [2], and, more recently, K-27 [38–40] and K-48 [40,41]. A recent paper reported quantification of pralidoxime in human urine by use of capillary zone electrophoresis [42]. Although ultraviolet absorbance has usually been used to monitor the separated peaks [5,30,43] or spots [24,25], other methods, for example autoradiography of radiolabelled compounds [44], electrochemical detection [45], and an on-line coupled electrospray ionization mass spectrometry (ESOI-MS) [40] have been found to be extremely sensitive tools for analysis of trace amounts of pyridinium aldoximes.

Metabolism converts xenobiotic compounds into less lipophilic, or even hydrophilic, analogues, thereby facilitating their elimination. Hepatic metabolism plays a major role in the metabolic transformation of parent drugs. Microsomal preparations mainly imitate liver metabolism, and in-vitro microsomal metabolism has become a very useful, economical, and high-throughput test of drug biotransformation.

Thin-layer chromatography (TLC) has been used for characterization of the lipophilicity of pralidoxime, obidoxime, K-27, and K-48. The results indicated obidoxime, K-27, and K-48 were hydrophilic in character whereas pralidoxime was lipophilic [25].

Our objective in this work was to develop an HPLC method for routine monitoring of K-27 and K-48 levels during microsomal treatment.

EXPERIMENTAL

Chemicals and Reagents

K-27 ((1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)propane dibromide) and K-48 (1-(4-hydroxyiminomethylpyridinium)-4-(4-carbomoylpyridinium)butane dibromide) were synthesized as described elsewhere [20] (Fig. 1). All other chemicals were obtained commercially in the best available quality. Disodium hydrogen phosphate dihydrate, citric acid monohydrate, ethylenediamine tetraacetic acid disodium salt dihydrate (Na_2EDTA), and acetonitrile were from Merck (Darmstadt, Germany), 1-octane sulfonic acid sodium salt, phosphoric acid, and trifluoroacetic acid from Sigma–Aldrich (St Louis, MO, USA), and 70% perchloric acid (PCA) from Fluka (Buchs, Switzerland). Water was double-distilled and deionized, and of HPLC grade.

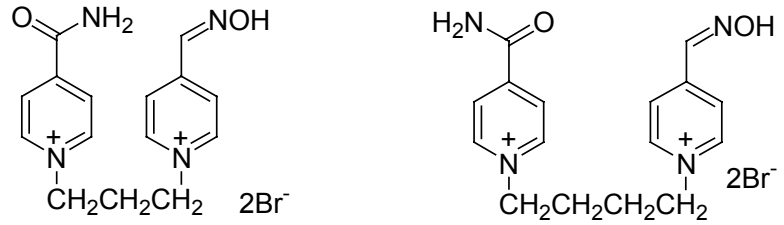

Fig. 1

The chemical structures of K-27 (left) and K-48 (right)

Microsomal Preparation

K-27 (22.1 μg mL^{-1}) and K-48 (22.9 μg mL^{-1}) were incubated for 45 min with rat liver microsomes (Gedeon Richter, Budapest, Hungary; lot. no. 060630/control; 0.5 mg mL^{-1}) in 2.0 mL incubation medium consisting of Tris-HCl buffer (0.12 mM, pH 7.4 at 37°C), $MgCl_2$ (5 mM), sodium pyrophosphate (6.25 mM), D-glucose 6-phosphate (5 mM), D-glucose 6-phosphate dehydrogenase (1 U mL^{-1}), and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH, 0.5 mM). Similar experiments were carried out using diabetic rat liver microsomal preparations. Incubation was terminated by addition of 70% ice-cold perchloric acid. HPLC analysis was performed after tenfold dilution of the samples. Each experiment was performed in triplicate.

Chromatography

Samples were analysed by reversed-phase high-performance liquid chromatography with electrochemical detection (HPLC–EC). Compounds were separated on a 250 mm × 4.6 mm, 5-μm particle size, Zorbax RX-C18 column protected by a 12.5 mm × 4.6 mm precolumn containing the same packing material, both from Kromat (Budapest, Hungary). The column temperature was 30°C. The mobile phase contained 56.2 mM Na_2HPO_4, 47.9 mM citric acid, 0.027 mM Na_2EDTA, 0.925 mM octane sulfonic acid, and acetonitrile–phosphate buffer 80:950 (*v*/*v*); the pH was adjusted to 3.7 by addition of 85% H_3PO_4. Samples were injected by means of an injector with a 50-μL loop and mobile phase was delivered at a flow rate of 1 mL min^{-1} by means of a Jasco (Tokyo, Japan) PU1580 pump.

Separations were monitored with an Intro amperometric detector (Antec; Leyden, Zoeterwoude, The Netherlands) at E_{ox} = 0.65 V, sensitivity of 10 nA, and a 0.1-s time filter. Chromatograms were electronically stored and evaluated by use of Borwin 1.21 chromatography software (JMBS, Le Fontanil, France).

HPLC was also performed with a C_{30} column, acetonitrile–water–trifluoroacetic acid 50:950:1 as mobile phase, and monitoring by UV absorbance at 286 nm.

Calibration

Calibration solutions in the range 5–3000 ng mL^{-1} were prepared by spiking 0.8 M PCA supernatant from the microsomal preparation with different amounts of K-48 stock solution; each solution was chromatogra-

phed in triplicate. Spiked samples were stored at −80°C. All concentrations were determined and found to be within 100 ± 5% of the original values within 36 h of preparation of the standard solution.

RESULTS

No interfering peaks were observed in HPLC on the octadecyl silica column, with electrochemical detection, of rat liver microsomal preparation containing K-48 (Fig. 2, top). The same peak for K-48 in the spiked microsomal preparation was also observed after incubation for 30 min (Fig. 2, bottom). Under the HPLC conditions used the cycle time was 30 min.

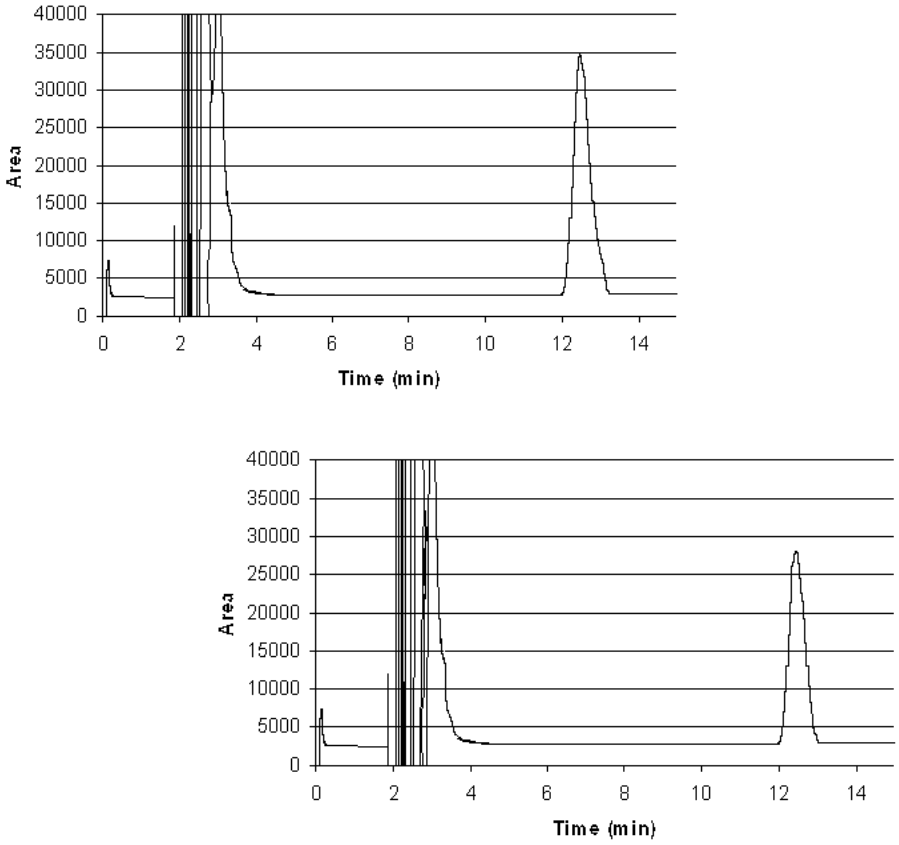

Fig. 2

Diabetic rat liver microsomal preparation spiked with 600 ng mL^{-1} K-48 (top) and the same microsomal preparation spiked with K-48 after 30 min incubation (bottom). The separation was monitored by use of electrochemical detection

The calibration plot obtained from the microsomal preparation spiked with different amounts of K-48 was linear, with R^2= 0.9959 (Fig. 3), and the method fulfilled the validation criteria. The limits of detection (*LOD*) and quantitation (*LOQ*) were 20 and 50 ng mL^{-1}, respectively. Both inter-day and intra-day reproducibility were within 2.5%.

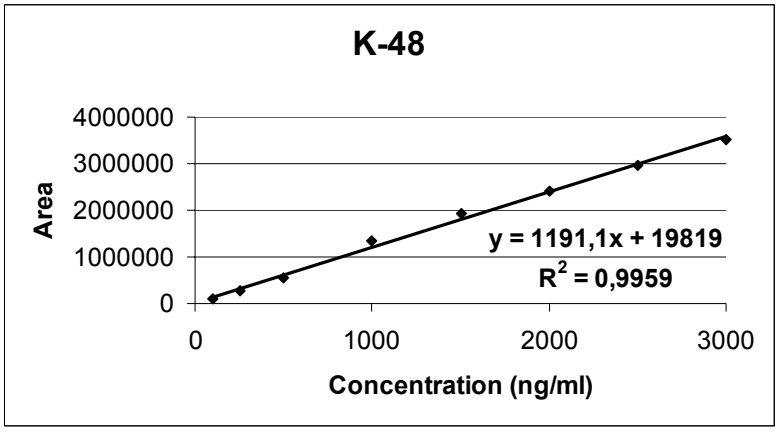

Fig. 3

Calibration plot obtained from rat liver microsomal preparation spiked with K-48. The separation was monitored by use of electrochemical detection

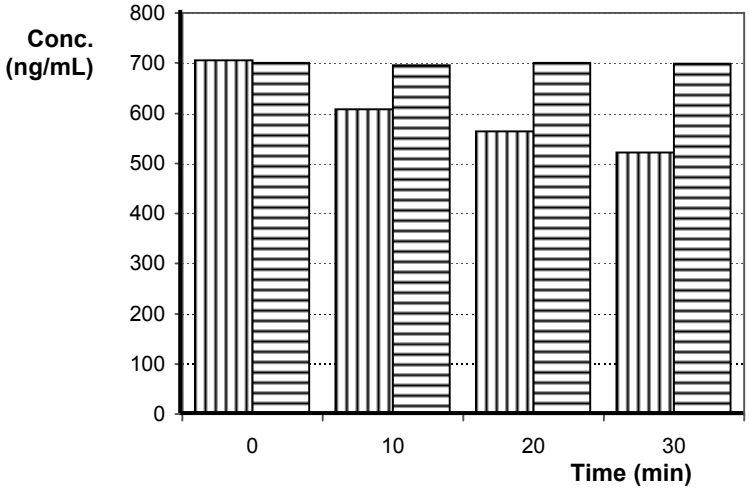

Fig. 4

Microsomal treatment of K-27 and K-48 resulted in different outcomes. K-48 was digested, and its concentration decreased (left column of each pair). K-27 (right column of each pair) resisted microsomal treatment and its concentration remained constant

Microsomal treatment for 30 min resulted in a decrease of approximately 20% in the concentration of K-48. There were no significant differences between the effects of microsomal preparation from control rats and diabetic rats. The progress of digestion is depicted in Fig. 4. K-27 was not digested by microsomal preparations that metabolized (consumed) K-48. Analysis by HPLC with electrochemical detection was suitable for monitoring changes in K-48 content throughout the treatment.

Improved separation was achieved by use of a C_{30} stationary phase. Use of ultraviolet absorbance monitoring at 286 nm also resulted in adequate sensitivity (Fig. 5)

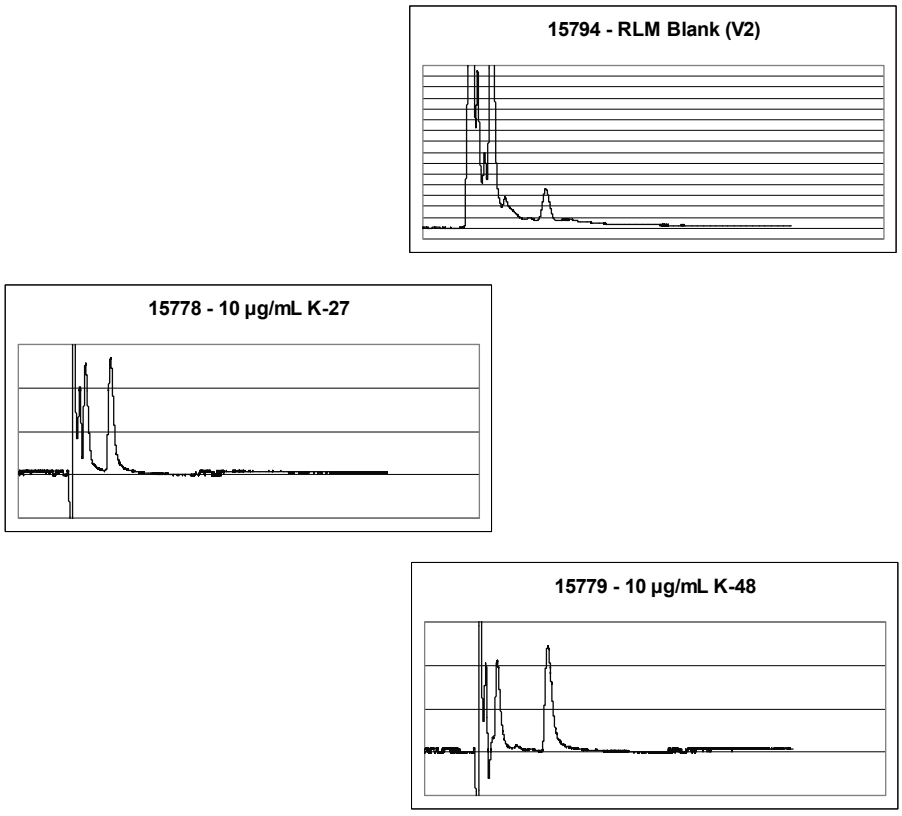

Fig. 5

Separation of K-27 and K-48 on C_{30} stationary phase with a mobile phase with a low organic modifier content (acetonitrile–water–trifluoroacetic acid 50:950:1). Detection was at 286 nm. The top chromatogram was obtained from rat liver microsomal preparation (RLM, blank). The centre chromatogram was obtained from RLM spiked with 10 µg mL^{-1} K-27. The bottom chromatogram was obtained from RLM spiked with 10 µg mL^{-1} K-48

DISCUSSION

Lipophilic drugs can penetrate biological membranes. Metabolism converts the drugs into more hydrophilic forms, thereby promoting excretion from the body. Drugs that are easily metabolized have been described as soft drugs whereas those that are metabolized to a very limited extent only, or not at all, are regarded as hard drugs (a review is available elsewhere [46]). Soft drugs can also be used as pro-drugs; these are metabolized to the active form in the target body compartment. Because hard drugs may not be significantly metabolized, they are not affected by the polymorphism of drug-metabolizing enzymes or the metabolizing capacity of the liver.

The presence of an intact –C=N–OH group in the reactivator structure is essential for organophosphate removal; hepatic first-pass metabolism (if present) might reduce or abolish the activity of such reactivator. Liver microsomal preparations have been widely used for preliminary in-vitro metabolic screening studies [47,48]. In-vitro work may save the lives of a large number of animals and enables variation of the experimental conditions according to requirements. Although mouse, rabbit, and other liver microsomal preparations have been used in the past, nowadays rat microsomes are preferred.

Both cytochrome P450 enzymes [47,49] and the flavin monooxygenase (FMO) enzyme system play a major role in drug metabolism. Wide inter-individual variation is observed for most of these enzymes, both because of genetic polymorphism and as a consequence of physiological or pathophysiological states, for example pregnancy, starvation, ascorbic acid deficiency, gonadectomy, and diabetes (a review is available elsewhere [46]). Xenobiotic compounds also affect the state of microsomal drug-metabolizing enzymes.

Isolated perfused rat liver has been used to yield metabolites of pralidoxime (abbreviated to 2-PAM in accordance with its chemical name). The rate of disappearance of 2-PAM was monitored using light absorbance at 340 nm after centrifugation and precipitation. Suggested metabolites included 1-methyl-2-cyanopyridinium and 1-methyl-2-pyridonecyanohydrin; another metabolite was assumed to be the O-conjugate.

As already mentioned above, the occurrence (or non-occurrence) of metabolism can be studied by subjecting a drug to microsomal preparations, a technique also known as ‘in-vitro’ metabolism. Analysis of the drug and its metabolites requires cell culture or subcellular fractions. Special condi-

tions can be examined by using microsomal preparations from disease-model animals (e.g. diabetic rats) [48]. The effect of diabetes on hepatic metabolism has been described by Borbás et al. [48]. Although results from in-vitro experiments do not always correlate with those from in-vivo studies, microsomal metabolism gives an indication of the major types of chemical alteration of drug molecules resulting from metabolism.

The major objective of microsomal metabolism is to check the resistance of a drug to oxidative metabolism. Any of the major separation or chromatographic methods can be used to monitor drug consumption. Reversed-phase HPLC has been shown to be suitable for monitoring the amount of a bisquaternary pyridinium aldoxime in different body compartments, and when a bisquaternary pyridinium aldoxime (obidoxime) was excreted after treatment. Capillary zone electrophoresis (CZE) can also be used to separate bisquaternary pyridinium aldoximes [42].

We used HPLC to monitor the effects of microsomal treatment. Conventional systems use ultraviolet absorbance at different wavelengths, for example 304, 288, 286, 280, or 228 nm. Advanced techniques include use of HPLC with on-line mass spectrometric (MS) detection. Use of single-ion monitoring (SIM) is complicated by the specific nature of K-27 and K-48, which have two quaternary nitrogen atoms in pyridinium rings forming a salt, e.g. a dibromide. Depending on the conditions used for HPLC and those in the ionization chamber of the MS a wide variety of ions might be present in the mass spectrum. When electrospray ionization mass spectrometry (ESI–MS) is used the most characteristic ions are at *m*/*z* 299 and 150. One of the molecular ions (*m*/*z* 299) is generated when the dibromide of K-48 (*m*/*z* 460) takes up one proton but repels the two bromide ions, and the two bromide ions also take two protons with them ($460 + 1 - (2 \times 80) - 2 = 299$). As a consequence the molecule has one positive charge. Another characteristic ion might also be generated at *m*/*z* 150, when the K-48 dibromide takes up one proton and loses two bromide ions and one proton ($460 + 1 - (2 \times 80) - 1 = 300$), resulting in two positive charges, and therefore giving an *m*/*z* value of 300/2 = 150. The ratio of these SIM values depends on the load and condition of the HPLC system. The situation in SIM is further complicated when the mass fragments contain ions from the buffer used (e.g. sodium, *m*/*z* 23) or from ion-pairing reagents, etc. For this reason hyphenated HPLC–MS has been used when screening for metabolites of K-48 only [40]. Although HPLC–MS enabled identification of the metabolites of microsome-treated K-48 [40], no metabolic alteration

of K-27 was found either in vitro (microsomal treatment) or in vivo (injected into rats; unpublished work).

Our recent experiments confirm the suitability of HPLC with electrochemical (amperometric) detection for analysis of bisquaternary pyridinium aldoximes, for example K-27 [39]. Reversed-phase HPLC with electrochemical detection is a sensitive and specific method and does not require use of hyphenated HPLC–MS.

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