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Determination of depleted uranium, pyridostigmine bromide and its metabolite in plasma and urine following combined administration in rats

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Abstract

A simple and reliable method was developed for the quantification of depleted uranium, the anti nerve agent drug pyridostigmine bromide (PB;3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) and its metabolite *N*-methyl-3-hydroxypyridinium bromide in rat plasma and urine. The method involved using solid phase extraction and spectrophotometric determination of uranium, and high performance liquid chromatography (HPLC) with reversed phase C_{18} column, and UV detection at 280 nm for PB and its metabolite. Uranium was derivatized using dibenzoylmethane (DBM) then the absorbance was measured at 405 nm. PB and its metabolite were separated using a gradient of 1–40% acetonitrile in 0.1% triflouroacetic acid water solution (pH 3.2) at a flow rate of 0.8 ml/min in a period of 14 min. Limits of detection were 2 ng/ml for uranium and 50 ng/ml for PB and its metabolite. Limits of quantitation were between 10 and 100 ng/ml for uranium and the other two analytes, respectively. Average percentage recovery of five spiked plasma samples were 83.7 ± 8.6, 76.8 ± 6.7, 79.1 ± 7.1, and from urine 82.7 ± 8.6, 79.3 ± 9.5 and 78.0 ± 6.2, for depleted uranium, PB and *N*-methyl-3-hydroxypyridinium bromide, respectively. The relationship between peak areas and concentration was linear for standards between 100 and 1000 ng/ml for all three analytes. This method was applied to analyze the above chemicals and metabolites following combined administration in rats.

Keywords: Gulf war veterans illness; Depleted uranium; Pyridostigmine bromide; Anti-nerve agent

1. Introduction

Depleted uranium is a by-product of the enrichment process of uranium [1]. During the Gulf War, depleted uranium has been used as a weapon [2]. The US Army used an estimated 9500 depleted uranium tank rounds during the Gulf War, either in training or in practice. As a result a number of US personnel were exposed to depleted uranium [3,4]. PB was used in the treatment of myasthenia gravis patients, and may be used following surgery in the reversal of neuro-

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muscular blockade [5,6]. PB has been used during the Gulf war to protect veterans against nerve agents such as Sarin and Soman [7–9]. PB inhibited acetylcholinesterase and butyrylcholinesterase enzymes in rats [9]. Depleted uranium produced tumors in human osteoblast cells in vitro [10], caused kidney damage in rats [11], produced adverse effects on the reproductive and central nervous systems [4], caused significant decrease in the pregnancy rate in mice [12], and enhanced mutagenic activity in *salmonela typhiurium* strain in urine samples from animals implanted with depleted uranium pellets [13]. Acute poisoning with depleted uranium elicited renal failure that could lead to death [14,15]. Depleted uranium is inhaled and absorbed through the skin and eyes [7,16]. Following its absorption, depleted uranium was distributed into tissues and eliminated in urine [17–21]. PB was reported to absorbed into plasma and excreted in urine following oral or intravenous dose in rats [22–24], in man [25,26], and in dogs [27]. Different methods have been used to determine uranium; spectrophotometric assays [28,36], radiochemical procedures [17], inductively coupled plasma-mass spectrometry [18,29], and high-precision kinetic phosphorescence analysis [19,21]. Methodologies for the determination of PB and its metabolite in biological matrices have been reported; assaying radioactivity following application of radiolabel compounds [25,37]. Limitation of this method is only can be used in laboratory animals, high performance liquid chromatography (HPLC) [27,30,31] with an advantage of simultaneous analysis of PB and its polar metabolites, gas chromatography [26,32,38], although this method is sensitive, it is not applicable for determination of polar metabolites, gas chromatography-mass spectrometry [33], and radioimmunoassay techniques [23].

We hypothesized that combined exposure to PB and depleted uranium could be resulted in toxic interactions. To examine possible pharmacokinetic interactions between PB and depleted uranium, a method is needed for the determination of these compounds in plasma and urine. This study reports on a method for the determination of uranium, PB and its metabolite in rat plasma and urine. The method was applied for the determination of these compounds following combined oral dose of PB and dermal dose of depleted uranium in rats.

2. Experimental

2.1. Chemicals and materials

Uranyl acetate dehydrated (depleted uranium) was obtained from Electron Microscopy Sciences (Fort Washington, PA, USA). Pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide, Fig. 1), dibenzoylmethane (DBM), trioctylphoshine oxide (TOPO) and tributyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-methyl-3-hydoxypyridinium bromide was prepared in our laboratory following the method by Somani et al. [25]. Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky, USA). C_{18} Sep-Pak^{R} cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA, USA).

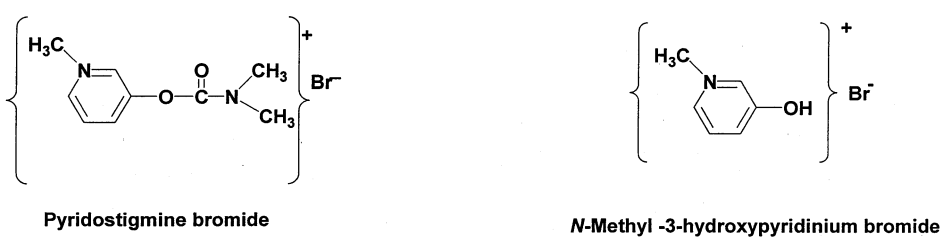

Fig. 1. Structures of pyridostigmine bromide and *N*-methyl-3-hydroxypyridine bromide.

2.2. Animals

Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). The animals were kept in plastic metabolic cages. Five rats were treated with a combination of a single dermal dose of 40 mg/kg of uranyl acetate (depleted uranium) and a single oral dose of 13 mg/kg of PB. Five untreated control rats were treated with water. Animal treatment and handling was conducted according to NIH and USDA guidelines. The animals were held in separate metabolic cages to allow collection of urine samples. They were kept in a 12 h light/dark cycle (temperature 21–23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) and tap water. Urine samples were collected from treated and control rats 24 h following dosing. The animals were anesthetized with halothane and sacrificed by heart exsanguinations. Blood was collected via heart puncture with a heparinized syringe and centrifuged at 2400 rpm for 15 min at 5°C to separate plasma. Urine and plasma samples were stored at −20°C prior analysis.

2.3. Instrumentation

The absorbance measurement of uranium was carried out with a Shimadzu UV-Visible-1601 spectrophotometer (Shimadzu Scientific Instruments, Inc., Norcross, GA, USA). The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, *a Waters Ultra WISP* 715 *autoinjector*, and a Waters 2487 Dual λ absorbance detector. A guard column (Supelco, 2 cm × 4.0 mm, 5 μm (Supelco Park, Bellefonte, PA), and a reversed-phase C_{18} column μBondapak™ C_{18} 125 Å 10 μm, 3.9 × 300 mm were used, (Waters Corporation).

2.4. Sample preparation

A 0.5 ml plasma and a 1 ml of urine samples from untreated rats were spiked with (100 μl) of concentrations ranging between 100 and 1000 ng/ml of depleted uranium, PB, and *N*-methyl-3-hydroxypyridinium bromide. Half of the spiked sample (300 μl of plasma and 550 μl of urine samples) was used for extraction of depleted uranium and the second part used for the determination of PB and its metabolite. Depleted uranium was extracted using a modified method of that described by Shamsipur et al. [28]. Urine samples that used for the determination of depleted uranium were diluted several times to reach the concentrations that fit within the standard calibration curve. Spiked and treated samples were acidified with 50 μl of 1 N acetic acid (pH 4). Disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges were conditioned with 5 ml of acetonitrile, then equilibrated using 5 ml of water prior use. For extraction of uranium, a solution of 50 mg/ml of TOPO in methanol was passed through the cartridge. The spiked urine and plasma samples were vortexed for 30 s, centrifuged for 5 min at 1000 g, and the supernatant was loaded into the disposable cartridges, then washed with 5 ml of water, and eluted two times by 2.5 ml of methanol and reduced in a marked tube to 500 μl using gentle stream of nitrogen, prior to analysis by HPLC or for spectrophotometric determination

2.5. Spectrophotometric determination

The solution of plasma or urine residues was transferred into a glass test tube containing 0.5 ml of DBM solution (2% w/v DBM in (1:1) pyridine-methanol solution and diluted to 3 ml using methanol. Concentration of depleted uranium (uranyl acetate) was determined spectrophotometrically at 405 nm.

2.6. Chromatographic conditions

A 10 μl aliquot of plasma or urine SPE elute was injected into HPLC using *autoinjector*. The mobile phases were water (adjusted to pH 3.2 using 1 N acetic acid) and acetonitrile. The mobile phases were filtered before use. The gradient started at 1% acetonitrile, increased linearity to 25% acetonitrile at 6 min, then increased linearity to 40% acetonitrile by 10 min. Then the system returned to 1% acetonitrile at 11 min where it was kept under this condition for 3 min to re-equili-

brate. The flow rate was 0.8 ml/min. The eluents were monitored by UV detection using a wavelength of 280 nm for PB and *N*-methyl-3-hydroxypyridinium bromide. The chromatographic analysis was performed at ambient temperature.

2.7. Calibration procedures

Five different calibration standards of a mixture of depleted uranium, PB and *N*-methyl-3-hydroxypyridinium bromide were prepared in methanol. Their concentrations ranged from 100 to 1000 ng/ml. Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of the concentration using GraphPad Prism program for windows (GraphPad Software, Inc., San Diego, CA, USA). The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

2.8. Accuracy and precision

Intra-day precision and accuracy of the method were determined in plasma and urine samples spiked with the analytes. For intra-day accuracy and precision, plasma and urine samples ($n = 5$) were spiked at concentrations of 100, 200, 400, 500 and 1000 ng/ml. The relative error percentage accuracy was determined as mean of detected concentration-added amount/added amount $\times$ 100. The coefficient of variation (CV) was calculated for the determination of precision.

2.9. Limits of detection (LOD) and limits of quantitation (LOQ)

Limits of detection and limits of quantitation were determined at the lowest concentration to be detected or quantify, taking into consideration a 1:3 and 1:10 signal to noise ratio, respectively. The LOQ was repeated five times for confirmation.

3. Results

3.1. Standard calibration curves

Calibration plots for all three analytes were linear over the concentration range (100–1000 ng/ml) with calibration coefficients (r^2) values of > 0.997.

3.2. Chromatogram

Chromatographic profiles were obtained for rat plasma and urine samples (Figs. 2 and 3). Retention times were 4.7 min and 10.2 min for PB and *N*-methyl-3-hydroxypyridinium bromide, respectively. Chromatogram of blank urine and plasma samples shows no interference from endogenous substances.

3.3. Extraction efficiency and recovery

Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates for concentrations between 100 and 1000 ng/ml (Table 1). Average percentage recoveries were 83.7 ± 8.6, 76.8 ± 6.7, and 79.1 ± 7.1 from plasma and 82.7 ± 8.6, 79.3 ± 9.5 and 78.0 ± 6.2 from urine for depleted uranium, PB and *N*-methyl-3-hydroxypyridinium bromide, respectively.

3.4. Accuracy and precision

Results of the intra-day accuracy and precision were calculated as described under Section 2.8. Average percentage relative error of accuracy for all concentrations added in plasma was 3.9 ± 0.8, 4.0 ± 1.3, $3.6 \pm 1.0\%$, and in urine samples was 3.1 ± 0.7, 2.5 ± 0.6, and $2.7 \pm 0.9\%$ for uranium, PB, and *N*-methyl-3-hydroxypyridinium bromide, respectively. Intra-day precision determined as percent coefficient of variation (%CV) for plasma and urine samples were ranged between 1.2 ± 0.8 and $2.3 \pm 0.9\%$.

3.5. Limits of detection

Blank plasma and urine samples from untreated rats were used as references for plasma and urine collections. Limits of detection were calculated from a peak signal to noise ratio of 3:1. The resulting detection limits were 2, 50 and 50 ng/ml

Table 1
Percent recovery of depleted uranium, pyridostigmine bromide and its metabolite from rat plasma and urine[a]

Concentration (ng/ml)	Plasma			Urine		
	Depleted uranium	Pyridostigmine bromide	*N*-methyl-3-hydroxypyridinium bromide	Depleted uranium	Pyridostigmine bromide	*N*-methyl-3-hydroxypyridinium bromide
1000	86.8 ± 6.4	80.1 ± 6.2	81.4 ± 6.8	90.2 ± 8.3	81.9 ± 11.8	79.4 ± 9.1
500	83.9 ± 9.5	79.5 ± 7.8	83.6 ± 6.9	83.6 ± 7.8	85.1 ± 8.4	84.2 ± 4.8
400	85.4 ± 10.3	77.2 ± 5.7	80.5 ± 7.7	82.9 ± 9.6	78.9 ± 9.2	80.1 ± 6.8
200	78.4 ± 8.4	72.6 ± 5.9	78.4 ± 8.2	80.2 ± 8.1	77.2 ± 8.8	76.1 ± 5.3
100	84.1 ± 8.6	74.6 ± 9.2	71.5 ± 5.8	76.4 ± 9.4	73.6 ± 9.1	70.2 ± 4.9

[a] Values are expressed as mean ± S.D. of five replicates.

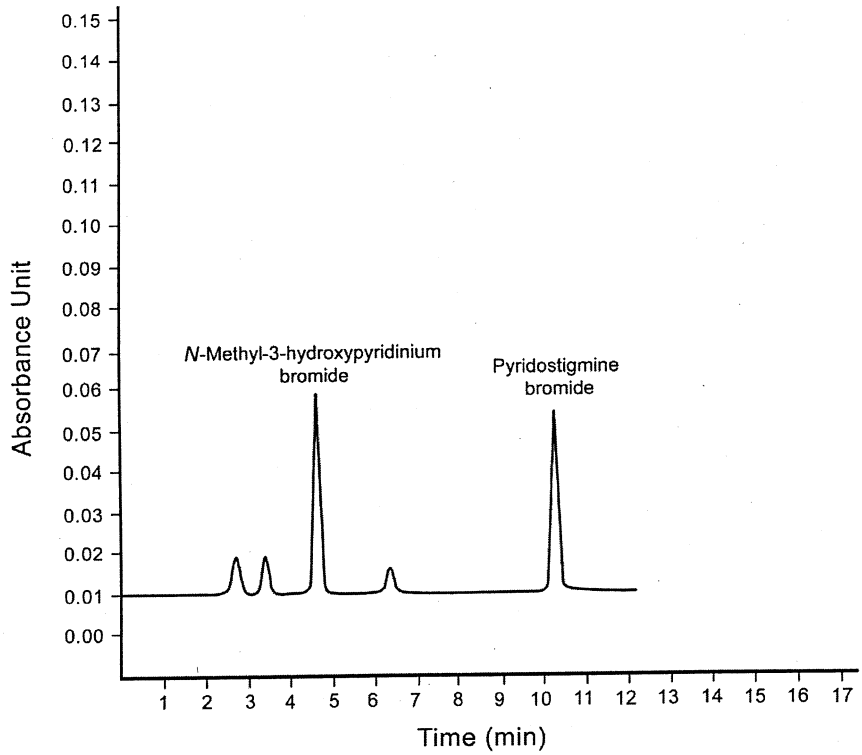

Fig. 2. Chromatogram of spiked plasma sample of pyridostigmine bromide and *N*-methyl-3-hydoxypyridinium bromide under established HPLC conditions.

for uranyl acetate (depleted uranium), PB, and *N*-methyl-3-hydroxypyridinium bromide, respectively.

3.6. Limits of quantitation (LOQ)

Limits of quantitation were determined at the lowest concentration to be quantify, taking into consideration 1:10 signal to noise ratio. They were determined be 10, 100 and 100 ng/ml for uranyl acetate (depleted uranium), PB and *N*-methyl-3-hydroxypyridinium bromide in plasma. In urine limits of quantitation were 5, 100, and 100 ng/ml for uranyl acetate (depleted uranium), PB and *N*-methyl-3-hydroxypyridinium bromide, respectively

3.7. Application of the method to biological samples

Following combined administration of a single dermal dose of depleted uranium and a single oral dose of PB the animals developed tremor, weakness, and several toxic symptoms. The rats were sacrificed 24 h following dosing. In plasma, depleted uranium and PB were detected. Their levels were 653 ± 287, and 273 ± 89 ng/ml, respectively. Depleted uranium and *N*-methyl-3-hydroxypyridinium have been identified in rat urine. Their concentrations were 1702 ± 296 and 179 ± 49 ng/ml, respectively. The concentrations were back calculated after serial dilutions to fit within the calibration curves.

4. Discussion

The present study reports the development of spectrophotometric and HPLC assays for the analysis of depleted uranium, PB and metabolites in rat plasma and urine following combined exposure.

Linearity of standard calibration curves for the chemicals in the present method is consistent with previous reports. Chan et al. [34] reported a linear range for PB in human plasma over concentrations between 50 and 1000 ng/ml.

Recoveries of the chemicals and metabolites were suitable for application of the method for analysis of treated samples for parent compounds and their metabolites. Low recovery for PB might have resulted from the instability of PB, in earlier study Aquilonius and Hartvig [6] reported that hydrolysis of PB could take place in buffer solutions, plasma and blood. Hennis et al. [27] reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, while Chan et al. [34] reported a recovery of 82% of PB from plasma. Recovery of the analyzed compounds in this method was between 78 and 84%. This range lies within the reported values in the literature, taking into consideration simultaneous analysis of the parent chemicals and their metabolites.

The limits of detection reported in our method allow analysis of samples from treated animals following doses similar to real human exposure. The ability to detect depleted uranium, PB in plasma, and depleted uranium and *N*-methyl-3-hydroxypyridinium bromide in urine after 24 h of dosing is evidence of the method suitability. Hennis et al. [27] reported a 50 ng/ml as a limit of detection of *N*-methyl-3-hydroxypyridinium in dog plasma using ion-exchange liquid chromatography. Miller and Verma [23] reported a 2.5 ng/ml

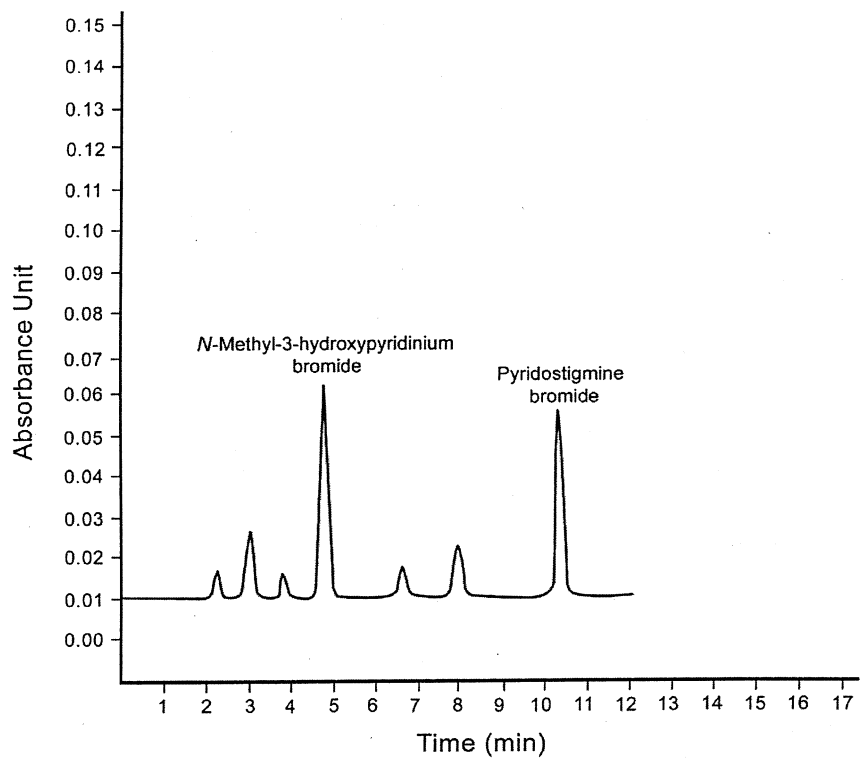

Fig. 3. Chromatogram of spiked urine sample with pyridostigmine bromide and *N*-methyl-3-hydoxypyridinium bromide under established HPLC conditions.

as detection limit of PB in tissues using radioimmunoassay method, while using HPLC technique, limits of detection of PB in plasma was 10 ng/ml [30], and ranged between 2.7 and 18.6 ng/ml in plasma using GC [34]. Limit of detection of uranium was varied according to method of analysis. It was 0.15 ng/l using ICP-MS [18], and 100 ng/l using spectrophotometric assay [28].

The method was applied successfully to analyze the chemicals and metabolites in plasma and urine. The detection of uranium in urine samples is in consistent with earlier studies that reported an average concentration of uranium in urine of subjects from a normal background environment was 12.8 ng/, with a possibility to detect 0.2 ng of uranium [35], while urinary levels of depleted uranium 7 years after first exposure of Gulf War veterans to depleted uranium ranged between 0.01 and 30.7 μg/g creatinine [4]. Pellmar et al. [21] detected urinary concentration of uranium between were 224 ± 32 ng/ml (low dose) $- 1010 \pm 87$ ng/ml urine (high dose), after implantation of depleted uranium in rats.

A simple UV and HPLC method was developed for the quantification of uranium, PB and metabolites in rat plasma and urine samples. The method could be used in the pharmacokinetics studies to assess distribution of the depleted uranium, PB and its metabolite in body tissues and fluids following combined exposure.

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