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A validated HPLC method for the determination of pyridostigmine bromide, acetaminophen, acetylsalicylic acid and caffeine in rat plasma and urine

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Abstract

A method was developed for the separation and quantification of the anti-nerve agent pyridostgmine bromide (PB;3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), the analgesic drugs acetaminophen and acetylsalicylic acid, and the stimulant caffeine (3,7-dihydro-1,3,7-trimethyl-1 H-purine-2,6-dione) in rat plasma and urine. The compounds were extracted using C_{18} Sep-Pak^R cartridges then analyzed by high performance liquid chromatography (HPLC) with reversed phase C_{18} column, and UV detection at 280 nm. The compounds were separated using gradient of 1–85% acetonitrile in water (pH 3.0) at a flow rate ranging between 1 and 1.5 ml/min in a period of 14 min. The retention times ranged from 8.8 to 11.5 min. The limits of detection were ranged between 100 and 200 ng/ml, while limits of quantitation were 150–200 ng/ml. Average percentage recovery of five spiked plasma samples were 70.9 ± 9.5, 73.7 ± 9.8, 88.6 ± 9.3, 83.9 ± 7.8, and from urine 69.1 ± 8.5, 74.5 ± 8.7, 85.9 ± 9.8, 83.2 ± 9.3, for pyridostigmine bromide, acetaminophen, acetylsalicylic acid and caffeine, respectively. The relationship between peak areas and concentration was linear over range between 100 and 1000 ng/ml. The resulting chromatograms showed no interfering peaks from endogenous plasma or urine components. This method was applied to analyze these compounds following oral administration in rats.

Keywords: Pyridostigmine bromide; Acetylsalicylic acid; Acetaminophen; Caffeine

1. Introduction

Pyridostigmine bromide is used for the treatment of myasthenia gravis patients, and has been used as an antidote against possible attack by organophosphate nerve agents [1,2]. Acetaminophen and acetylsalicylic acid are widely used analgesic drugs, while caffeine is a well-known simulator. Combined exposure of veterans or patients to these chemicals is possible. Published reports showed that exposure to pyridostigmine bromide, the insect repellent DEET (*N*,*N*-diethyl-*m*-toluamide) and the insecticide permethrin enhanced neurotoxicity in hens [3], and increased lethality in rats [4].

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Pyridostigmine bromide was reported to absorbed into plasma and excreted in urine in rats [1,5–7] and in human [8,9]. Absorption and excretion of acetaminophen was rapid after oral application in human [10,11]. Acetylsalicylic acid and caffeine were also reported to be absorbed into plasma and excreted in urine following oral dose in human [12–14].

Analytical methods have been used for the determination of these compounds, when administered as individuals in biological matrices. These methods used high- performance liquid chromatography for analysis of acetylsalicylic acid [15–18], caffeine [14,19–21], pyridostigmine bromide [5,22,23], and acetaminophen [11,24,25], high- performance liquid chromatography–mass spectrometry [26], gas chromatography [10,27], gas chromatography–mass spectrometry [13], micellar electrokinetic chromatography (MEKC)[28], electrophoresis with paper chromatography [8], radioimmunoassay [7], spectrophotometric analysis [29], and capillary electrophoresis [12]. The reported limits of detection of these compounds in plasma or urine samples using HPLC-UV ranged between 10 and 300 ng/ml. Their recoveries were between 80 and 100% [14–16,23,30].

We hypothesized that combined exposure to these compounds could be resulted in toxic interactions. We plan to study the pharmacokinetic profile of each compound, alone and in combination. To achieve this objective, a method is needed for the simultaneous determination of pyridostigmine bromide, acetylsalicylic acid, acetaminophen, and caffeine. This study describes a method for simultaneous determination of these chemicals in rat plasma and urine using solid phase extraction coupled with reversed phase-high performance liquid chromatograph.

2. Experimental

2.1. Chemicals and materials

Pyridostigmine bromide (98% PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), acetaminophen (99%), acetylsalicylic acid (99.5%), and caffeine (3,7-dihydro-1,3,7-trimethyl-1 H-purine-2,6-dione) (Fig. 1) were obtained from Sigma Chemical Co. (St Louis, MO). Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker Inc. (Paris, Kentucky, USA). C_{18} Sep-Pak^R cartridges were obtained

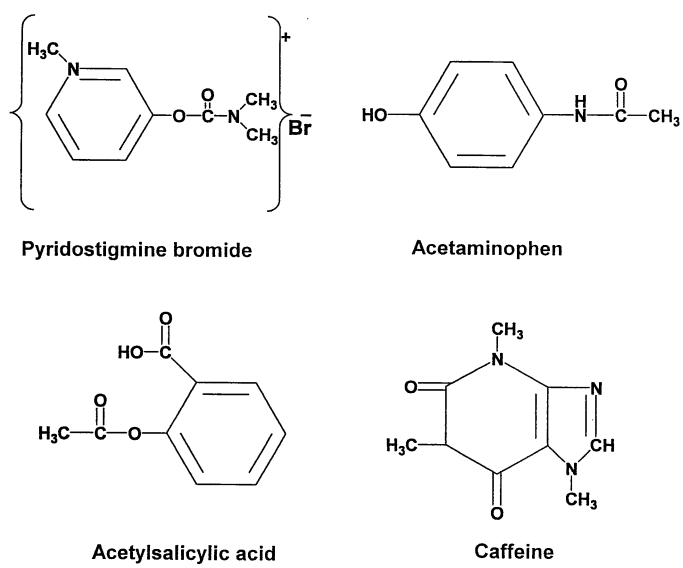

Fig. 1. Chemical structures of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine.

from Waters Corporation (Waters Corporation, Milford, MA).

2.2. Animals

Rats (Sprague–Dawley) were purchased from Zivic Miller (Zelienople, PA). Five rats were treated with a combination of a single oral dose of 1.3 mg/kg of pyridostigmine bromide, 5 mg/kg of acetaminophen, 5 mg/kg of acetylsalicylic acid, and 1 mg/kg of caffeine. Five control rats were treated with oral dose of water. The animals were held in metabolic cages to allow for the collection of urine samples. Urine samples were collected from treated and control rats 4 h after dosing, then the animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) 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 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 (Waters Corporation, Milford, MA). 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 A° 10 μm, 3.9 × 300 mm were used, (Waters Corporation, Milford, MA)

2.4. Sample preparation

A 0.2 ml plasma and urine samples from untreated rats were spiked with concentrations ranging between 200 and 1000 ng/ml of each of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine. Spiked and treated samples were acidified with 1 N acetic acid (pH 5.0). Disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges (Waters Corporation, Milford, MA) were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior use. 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 2 ml of water, and eluted twice by 2 ml of methanol and reduced to 500 μl using a gentle stream of nitrogen prior to analysis by HPLC.

2.5. Calibration procedures

Five different calibration standards of a mixture of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine were prepared in water. 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). The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

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

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

3. Results

3.1. Standard calibration curves

The standard calibration curves of peak area against concentration of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine are shown in Fig. 2. Linearity of the calibration curves for the four compounds was achieved for concentrations between 100 and 1000 ng/ml.

3.2. Chromatogram

Chromatographic profiles were obtained for rat plasma and urine samples after solid phase extraction using C_{18} Sep Pak^{R} cartridges under HPLC conditions, described above (Fig. 3 and Fig. 4).

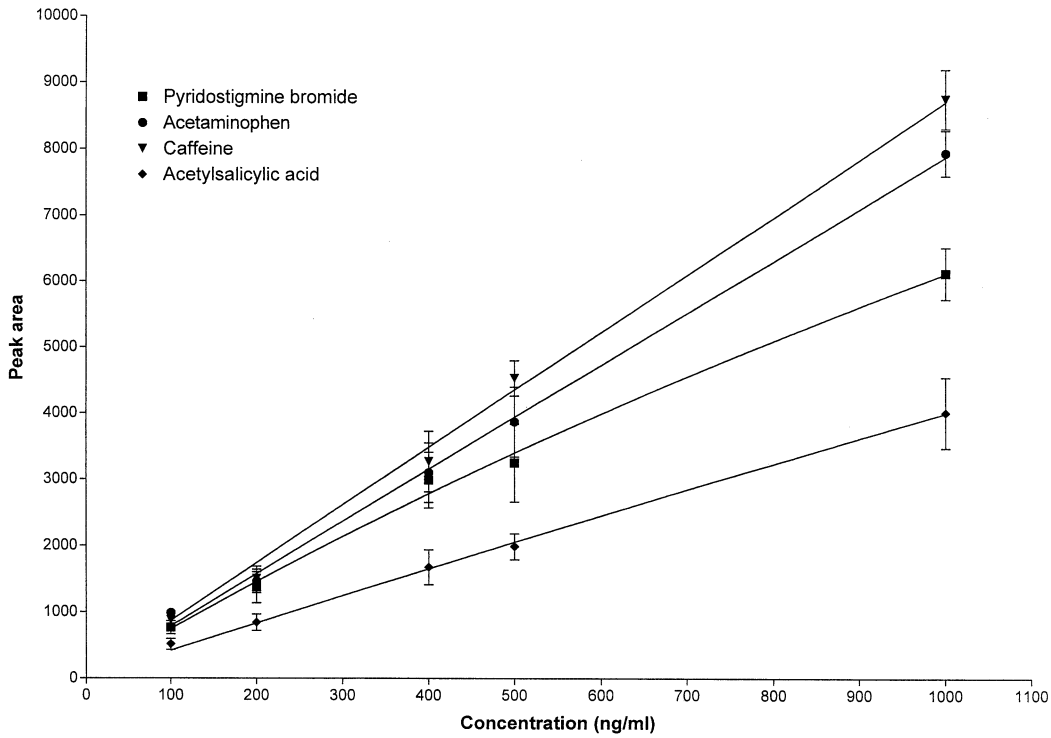

Fig. 2. Standard calibration curves of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine.

Retention times were 8.8, 9.9, 10.4, and 11.5 min for, acetaminophen, pyridostigmine bromide, caffeine and acetylsalicylic acid, respectively. The total run time was 14 min. The compounds were detected at 280 nm. Clean chromatogram shows no interference from endogenous substances in plasma and urine samples.

3.3. Extraction efficiency and recovery

The average percentage extraction recoveries of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine were determined at concentrations ranged between 200 and 1000 ng/ml (Table 1). Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recoveries were 70.9 ± 9.5, 73.7 ± 9.8, 88.6 ± 9.3, 83.9 ± 7.8, and from urine 69.1 ± 8.5, 74.5 ± 8.7, 85.9 ± 9.8, 83.2 ± 9.3, for pyridostigmine bromide, acetaminophen, acetylsalicylic acid and caffeine, respectively.

3.4. 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 range were 100, 100, 200, and 100 ng/ml for pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine, respectively.

3.5. Limits of quantitation (LOQ)

Limits of quantitation were determined to be 150 ng/ml for pyridostigmine bromide and acetaminophen, and 100 ng/ml for caffeine, and 200 ng/ml for acetylsalicylic acid in plasma. In urine limits of quantitation were 200, 150, 200, and 200 ng/ml for pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine, respectively.

Table 1
Percentage recovery of pyridostigmine bromide, acetaminophen, acetylsalicylic acid and caffeine from rat plasma and urine

Concentration (ng/ml)	Plasma				Urine			
	Pyridostigmine bromide	Acetaminophen	Acetylsalicylic acid	Caffeine	Pyridostigmine bromide	Acetaminophen	Acetylsalicylic acid	Caffeine
200	69.5 ± 9.6	65.1 ± 12.2	86.1 ± 10.2	73.9 ± 5.4	63.2 ± 11.9	72.1 ± 10.3	83.4 ± 7.6	80.1 ± 6.3
400	67.3 ± 10.1	73.6 ± 10.9	83.3 ± 12.7	81.2 ± 6.3	68.1 ± 7.2	75.4 ± 9.8	89.1 ± 11.9	78.2 ± 9.7
500	73.7 ± 8.3	78.1 ± 7.6	89.5 ± 9.6	89.6 ± 8.1	69.6 ± 8.1	72.3 ± 6.8	87.6 ± 8.5	83.3 ± 11.2
800	71.9 ± 11.2	76.3 ± 8.3	92.7 ± 7.3	87.2 ± 11.4	73.1 ± 6.9	78.4 ± 7.1	86.4 ± 12.1	86.1 ± 9.2
1000	72.1 ± 8.3	76.5 ± 10.2	91.4 ± 6.8	87.6 ± 7.8	71.2 ± 8.2	74.3 ± 9.3	82.9 ± 9.1	88.7 ± 10.2

Values are expressed as mean ± SD of five replicates.

3.6. Application of the method to biological samples

In order to validate the method, the method was used for the determination of the compounds in treated rat plasma and urine samples 4 h after dosing. In plasma, their levels were 253 ± 93, 1043 ± 291, 1440 ± 376 and 325 ± 146 ng/ml for pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine, respectively (Fig. 5). Their levels in urine were 12.5 ± 3.6, 23.8 ± 6.8, and 34.6 ± 11.9 μg/ml for pyridostigmine bromide, acetaminophen and caffeine, respectively (Fig. 6). Acetylsalicylic acid was not detected in the urine 4 h after dosing.

4. Discussion

This study reports on development of an HPLC method for determination of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine in plasma and urine of treated rats. Linearity of the present method is consistent with previous reports; Chan et al. [31] reported a linear range for pyridostigmine bromide in human plasma for concentrations between 50 and 1000 ng/ml. The curve was linear for concentrations between 0.18 and 10 mμmole/l of acetylsalicylic acid [16]. Vilchez et al. [29] obtained a linear calibration curve for concentrations between 100 and 1000 μg/ml of acetaminophen.

The chromatogram obtained following solid phase extraction and HPLC analysis shows no interference from plasma and urine components, indicating an efficient clean up method used and selectivity of the method. Recoveries of the chemicals were suitable for application of the method for analysis of treated samples for parent compounds following real-life exposure conditions. Low recovery for pyridostigmine bromide might have resulted from the use of solvent system that was not quite suitable for extracting pyridostigmine bromide, and at the same time it was needed

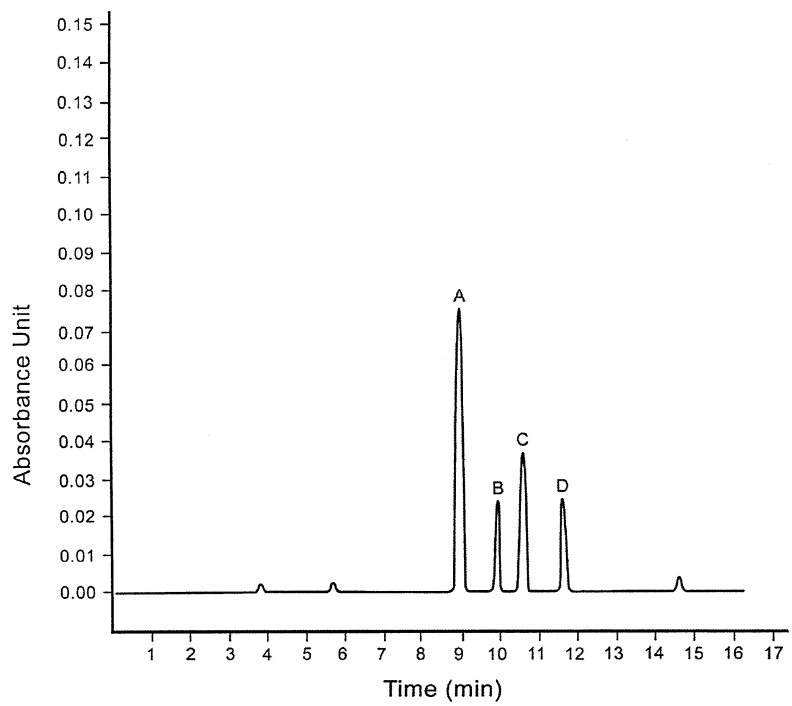

Fig. 3. Chromatogram of spiked plasma sample with (A) pyridostigmine bromide, (B) acetaminophen, (C) caffeine, (D) acetylsalicylic acid under described HPLC conditions.

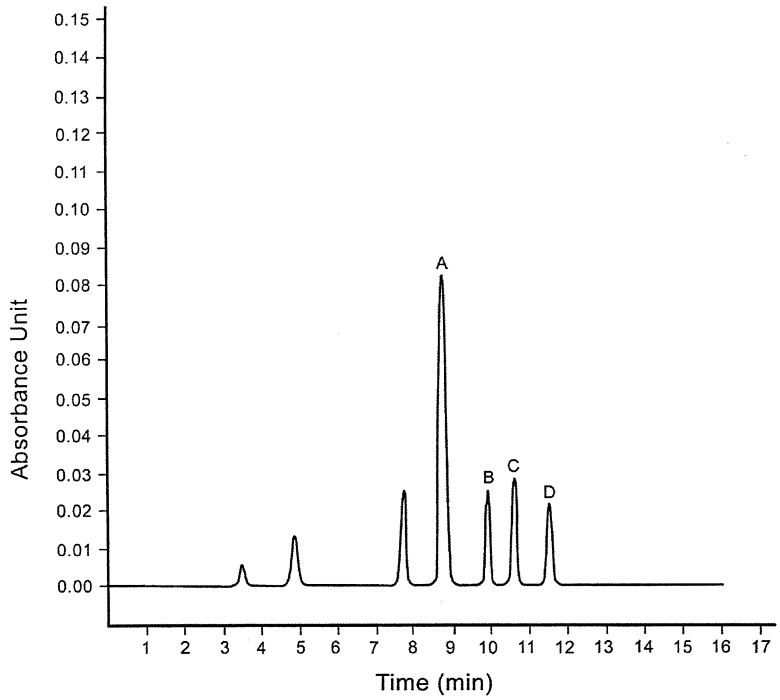

Fig. 4. Chromatogram of spiked urine sample with (A) pyridostigmine bromide, (B) acetaminophen, (C) caffeine, (D) acetylsalicylic acid under described HPLC conditions.

for extracting and analyzing the other three chemicals under similar conditions. In previous study Aquilonius and Hartvig [32] reported the difficulty of extraction and analysis of pyrdostigmine bromide, because of its in vitro hydrolysis could take place in buffer solutions, plasma and blood. Chan et al. [31] reported a recovery of 82% of pyridostigmine bromide from plasma at low concentration of 50 ng/ml, while its recovery was 92% when a concentration of 400 ng/ml was used Recoveries of acetaminophen, acetylsalicylic acid and caffeine from serum and urine were reported to be between 90 and 100% [16,24,30]. Recoveries of the chemicals analyzed in our method were between 65 and 93%. This range lies within the reported values in the literature, taking into consideration the simultaneous determination of the compounds.

The ability to detect the four compounds in plasma after 4 h of dosing is an evidence of the method applicability. Pyridostigmine bromide, acetaminophen, and caffeine were also detected in urine samples 4 h after dosing, while the failure to detect acetylsalicylic acid in urine might due to its rapid metabolism and conjugation. Limits of detection of the compounds in this method were

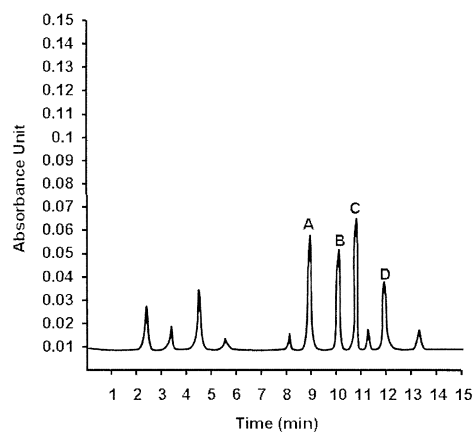

Fig. 5. Chromatogram of treated plasma sample with (A) pyridostigmine bromide, (B) acetaminophen, (C) caffeine, (D) acetylsalicylic acid under described HPLC conditions.

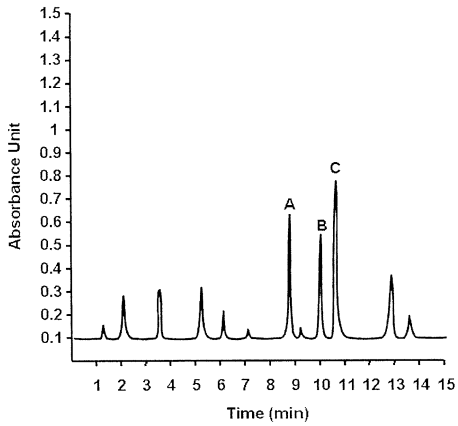

Fig. 6. Chromatogram of treated urine sample with (A) pyridostigmine bromide, (B) acetaminophen, and (C) caffeine under described HPLC conditions.

between 50 and 200 ng/ml, while limits of quantitation ranged between 150 and 200 ng/ml. In previous studies, limits of detection of caffeine and metabolites in biological fluids of monkeys was 100–300 ng/ml using HPLC [14], While it was 50 ng/ml of acetaminophen in serum [30], and was 200 ng/ml in children plasma, urine and saliva using HPLC [15]. Limits of quantitation of acetylsalicylic acid from human plasma and urine were 5 and 25 μg/ml using non-aqueous capillary electrophoresis [12], and it was 200 pg/ml from human plasma using GC-MS/MS [13]. Limits of detection of caffeine in human plasma was 10 ng/ml using HPLC [21] and was 5 ng/ml in human plasma and urine using HPLC-MS [26]. Using HPLC technique, limit of detection of pyridostigmine bromide in plasma was 10 ng/ml [22]. The reported limits of detection in the literature are consistent with our results for the simultaneous analysis of the four compounds that ranged between 100 and 200 ng/ml.

A rapid and simple HPLC method was developed for separation and residual analysis of pyridostigmine bromide, acetaminophen, acetylsalicylic acid, and caffeine in rat spiked and treated plasma and urine samples. Solid phase extraction was used which selectively extracted the above chemicals from plasma and urine samples without interference of an expected mixture of metabolites and endogenous compounds. The method could be used in the pharmacokinetics studies to assess distribution of the parent compounds in body tissues and fluids following combined administration. The main advantage of the method is the ability to analyze simultaneously the four compounds under similar conditions.

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