HPLC Analysis of Metabolically Produced Formaldehyde

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Abstract

Radiolabelled (–)-deprenyl is orally administered to rats, and urinary elimination of radiolabelled formaldehyde is detected. The separation is performed using high-performance liquid chromatography on octadecyl-silica stationary phase. Both the radioactivity and the UV absorbance of the dinitrophenylhydrazine formaldehyde peak are determined. Formaldehyde generation takes place by N-demethylation. Low levels of formaldehyde may have a beneficial role in counterbalancing the oxidative stress of the everyday person's life.

Introduction

Chromatography is the major procedure for therapeutic drug monitoring. The primary interest is focused on the level of the parent drug, and its blood level and urinary elimination are frequently analyzed.

Aldehydes can be preferentially and selectively determined by the use of chromatography. Gas chromatography (GC) had been employed to determine formaldehyde, acetaldehyde, propionaldehyde, and butyraldehyde, either in their free form or their adducts with dimedone (formaldemedone, acetaldemedone, propionaldemedone, and butyraldemedone) (1). These compounds were stable under the condition of GC. At the same time, the widespread use of liquid chromatography (LC) offered an easier and rather precise method for the quantitation of formaldehyde and the other aldehydes.

Thin-layer chromatography (TLC) offers an adequate tool to determine the formaldehyde level in biological samples. The stationary phase (the TLC plate) is disposable, therefore either no clean-up or only a simplified one is required prior to the analyses. Formaldehyde is reacted with dimedone forming formaldemedone, which has an intensive absorbance at 254 nm. Therefore formaldemedone can be easily detected using silica gel F_{254} plates. The dark spots of formaldemedone on the fluorescent background can be either easily observed visually, or they can be quantitatively evaluated using a densitometer (2–4).

High-performance LC (HPLC) determination of aldehydes is generally used when various body fluids are the subject of the study. Derivatizing agents should be used in the case of aliphatic aldehydes, such as formaldehyde having no chromophore part of the molecule. Both 1,3-cyclohexanedione (5), and 4-(N,N-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole (6) derivatives can be monitored using a fluorescence detector. Formaldehyde derivatives can also be monitored in the UV region with the use of various reagents, such as dimedone or 2,4-dinitrophenylhydrazine. The formaldehyde derivative with 2,4-dinitrophenylhydrazine is preferred for HPLC to any other reagents because it can be monitored in the region of 330–360 nm (7–9), that is more specific than monitoring the formaldemedone at 254 nm. Deng and Yu simultaneously determined formaldehyde and methylglyoxal in rat urine (10); formaldehyde was derivatized with 2,4-dinitrophenylhydrazine.

Recent publications have proven the role of cytochrome P450-dependent N-dealkylation in the biotransformation of deprenyl, in which cytochrome P450 2E1 (CYP2E1) played the major role while the role of CYP2B was minor (11). Two important metabolites of (–)-deprenyl have recently been recognized: formaldehyde and deprenyl-N-oxide. Determination of the N-oxide of (–)-deprenyl has been published (12). However, identification and quantitation of the metabolically generated formaldehyde has not been evaluated in an appropriate way. Recent papers reported on the total amount of formaldehyde in various body fluids, but neither their origin from (–)-deprenyl nor the dose dependency of the formed formaldehyde has been proven.

Our recent publication has given evidence of the formaldehyde production by metabolic N-demethylation (4). An HPLC method for the quantitative determination of the produced formaldehyde is described here.

Experimental

Materials

Chemicals and solvents [such as 2,4-dinitrophenylhydrazine (DNPH)] methanol, dimethyl sulfoxide, formic acid, and propionaldehyde were purchased from commercial sources in the highest available purity. Acetonitrile and water were of HPLC grade.

Solid-phase extraction (SPE) was carried out using either Discovery DSC-18 containing 100 mg of octadecyl silica (Supelco, Bellefonte, PA) or LiChrolute columns for SPE RP-18, 200-mg

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(Merck, Darmstadt, Germany).

L-Deprenyl [(–)-*N*-methyl-*N*-propynyl-1-methyl-2-phenyl-ethylamine hydrochloride] was a kind gift from Sanofi-Synthelabo [(formerly, Chinoin), Budapest, Hungary]. Radiolabelled L-deprenyl, [(–)-[14]C-*N*-methyl-*N*-propynyl-phenyl-isopropylamine, 932 MBq/mmol; 3.6 MBq/mg] was prepared from (L)-*N*-propynyl-phenyl-isopropylamine The radiolabelled L-deprenyl was prepared and supplied by the Institute of Isotopes Co, (Budapest, Hungary).

Treatment of animals

(–)-Deprenyl was administered p.o. (per os, through the mouth) to male rats of 200–240-g weights. The rats were kept in
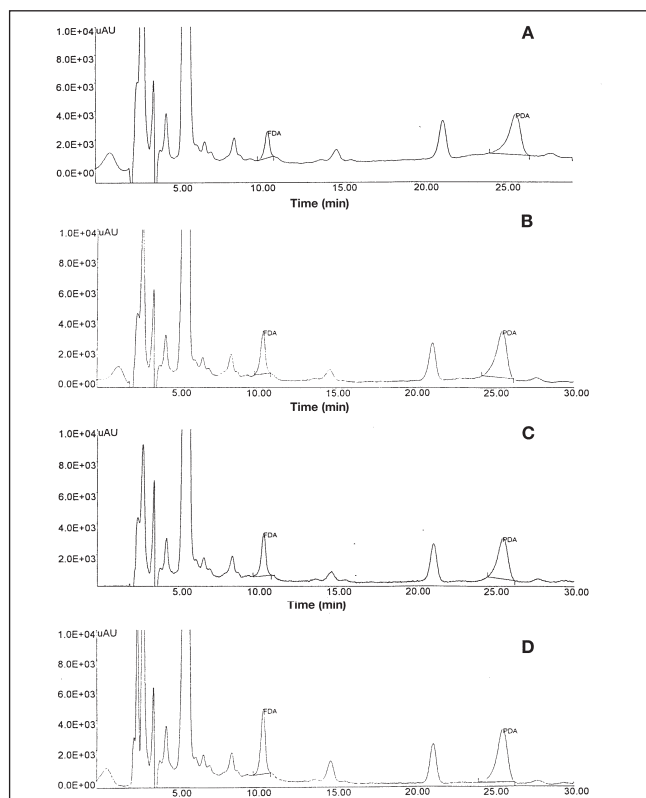
Figure 1. HPLC separation of urine samples after clean-up using SPE. The determined chromatograms indicate formaldehyde–DNPH (FDA) and propionaldehyde-DNPH (PDA) peaks of control rat urine (A), urine sample of a rat treated with 0.5 mg/kg (–)-deprenyl (B), urine sample of rat treated with 5 mg/kg (–)-deprenyl (C), and urine sample of rat treated with 50 mg/kg (–)-deprenyl (D).

special cages where an unlimited supply of food and water was provided, and urine was collected. The care, treatment, and sacrificing of the animals were performed following the Animal Care Ethical Codex of Semmelweis University (Budapest, Hungary).

HPLC

A JASCO (Tokyo, Japan) HPLC system containing a pump, injector, and diode-array UV detector was used. A 250- × 4.6-mm HPLC column containing 5 µm of Zorbax Rx-C18 as the stationary phase (Agilent Technologies, Palo Alto, CA) was applied for separation. The mobile phase contained acetonitrile–tetrahydrofuran–aqueous phosphate buffer [0.2 M Na_2HPO_4–0.1M citric acid (6.44:13.56) McIlvain buffer at pH 3.6], (40:5:55) using a flow rate of 1 mL/min. A 20-µL aliquot was injected into the HPLC system, and the chromatogram was monitored at 356 nm. The cycle time was 30 min.

HPLC–mass spectrometry

A Finnigan LCQ Advantage HPLC iontrap mass spectrometry (MS)–MS system (Thermo Finnigan, San Jose, CA) was used. A 55- × 4-mm HPLC column packed with Purospher STAR RP-18E (Merck, Darmstadt, Germany) stationary phase was employed for separation. The mobile phase was acetonitrile–water (52.5:47.5, v/v), and the pH was adjusted to 3.5 using formic acid. The mobile phase flow rate was 1 mL/min. The chromatograms (from the top to the bottom) were monitored using UV at 330 nm: total-ion current 100 trough, 1000 MS; 208.5–209.5 MS; 250.5–251.5 MS; 236.5–237.5 MS; 232.5–233.5 MS; and 222.2–223.5 MS for all compounds (twice, in UV and MS); formaldehyde–DNPH; methyl-glyoxal–DNPH; propionaldehyde–DNPH; propynylaldehyde–DNPH; and acetaldehyde–DNPH, respectively.

Methods

The urine was collected for 0–5 h and 5–24 h. A propionaldehyde internal standard (250 ng/sample) was added and mixed, and 250 g DNPH was given from a solution containing 2 mmol DNPH in dimethyl sulfoxide (DMSO) and acetate buffer [0.2M acetic acid–0.2M sodium acetate (16.4:3.6) Wolpole buffer at pH 4] (9:16, v/v). The mixture was held for 10 min at room temperature and then subjected to SPE. The octadecyl silica of SPE was conditioned with 2 mL methanol followed by 2 mL water. The sample was 0.5 mL and washed with 1 mL of water and 1 mL water–methanol (1:1) to elute the majority of the DNPH reagent. Next, formaldehyde–DNPH was eluted with 2-mL methanol. It was dried under nitrogen stream at 37°C, then dissolved in 200 µL acetonitrile. In each HPLC separation, a 20-µL sample was injected.

The effluent fractions were collected (0.5 mL each). An aliquot of each fraction was mixed with Ultima Gold cocktail (Packard Bioscience, Groningen, the Netherlands), and the radioactivity was determined in disintegration per minutes (dpm).

Results

Radiolabelled (–)-deprenyl was administered to

Table I. Urinary Elimination of Radioactivity after ^{14}C-Methyl-(–)-Deprenyl Was p.o. Administered to Rats

Dose of (–)-deprenyl	Total radioactivity administered (dmp/rat)	Urinary elimination of radioactivity			
		0–5 h	5–24 h	0–24 h	%Urinary elimination
0.5 mg/kg	2.17×10^7	8.58×10^5	6.78×10^6	7.638×10^6	35
5 mg/kg	2.17×10^8	1.36×10^7	6.12×10^7	7.48×10^7	34
50 mg/kg	2.17×10^9	1.72×10^8	4.07×10^8	5.79×10^8	26

rats in the doses of 0.5, 5.0, and 50 mg/kg body weight. Urine was collected in the first 5 h and for the following 19 h.

Propionaldehyde was used as the internal standard, and the aldehydes were reacted with 2,4-dinitrophenylhydrazine to form their 2,4-dinitrophenylhydrazone. The samples were subjected to HPLC separation on the octadecyl-silica stationary phase (Figure 1). The peaks belonging to both the DNPH–formaldehyde and DNPH–propionaldehyde were evaluated using their UV absorbance at 356 nm, and the effluent belonging to the peak of DNPH–formaldehyde was also collected. Quantitative evaluation was based on counting the radioactivity. The results are shown in Tables I–III.

Approximately 20 million dpm/rat was injected, and over 1000 dpm was recovered from one tenth of the urine when the pharmacological dose (0.5 mg/kg) of radiolabelled (–)-deprenyl was administered to rats. Considering the approximate 50% recovery during the treatment of urine, the (–)-deprenyl to formaldehyde conversion can be estimated to be approximately 0.01%.

In other experiments, the peak belonging to DNPH–formaldehyde was subjected to HPLC–MS–MS analysis. The results have proven that even a short column can give adequate virtual separation if a specific detection is used (Figure 2).

Table II. Urinary Elimination of Formaldehyde After p.o. Administration of (–)-Deprenyl to Rats
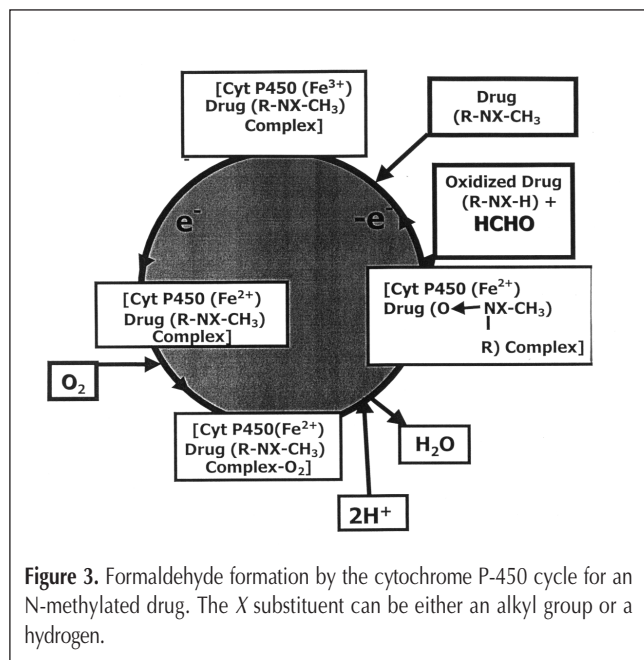
Dose of (–)-deprenyl	Urinary elimination of formaldehyde (ng) through the period of		
	0–5 h	5–24 h	0–24 h
0 mg/kg (control)	210	750	960
0.5 mg/kg	330	820	1150
5 mg/kg	168	1414	1582
50 mg/kg	666	1856	2522

Table III. Radioactive Formaldehyde in Each HPLC Run Originated From ^{14}C-Methyl-(–)-Deprenyl That Aas p.o. Administered to Rats

Dose of (–)-deprenyl	Dose of (–)-deprenyl (µmol/rat)	Total radioactivity administered (dmp/rat)	Formaldehyde (dpm) in each HPLC run			Eliminated formaldehyde (%)		
			0–5 h	5–24 h	0–24 h	0–5 h	5–24	0–24 h
0.5 mg/kg	0.448	2.17×10^7	680	640	1320	52	48	100
5 mg/kg	4.48	2.17×10^8	1130	3610	4740	24	76	100
50 mg/kg	44.8	2.17×10^9	3060	25.500	28.560	10.7	89.3	100
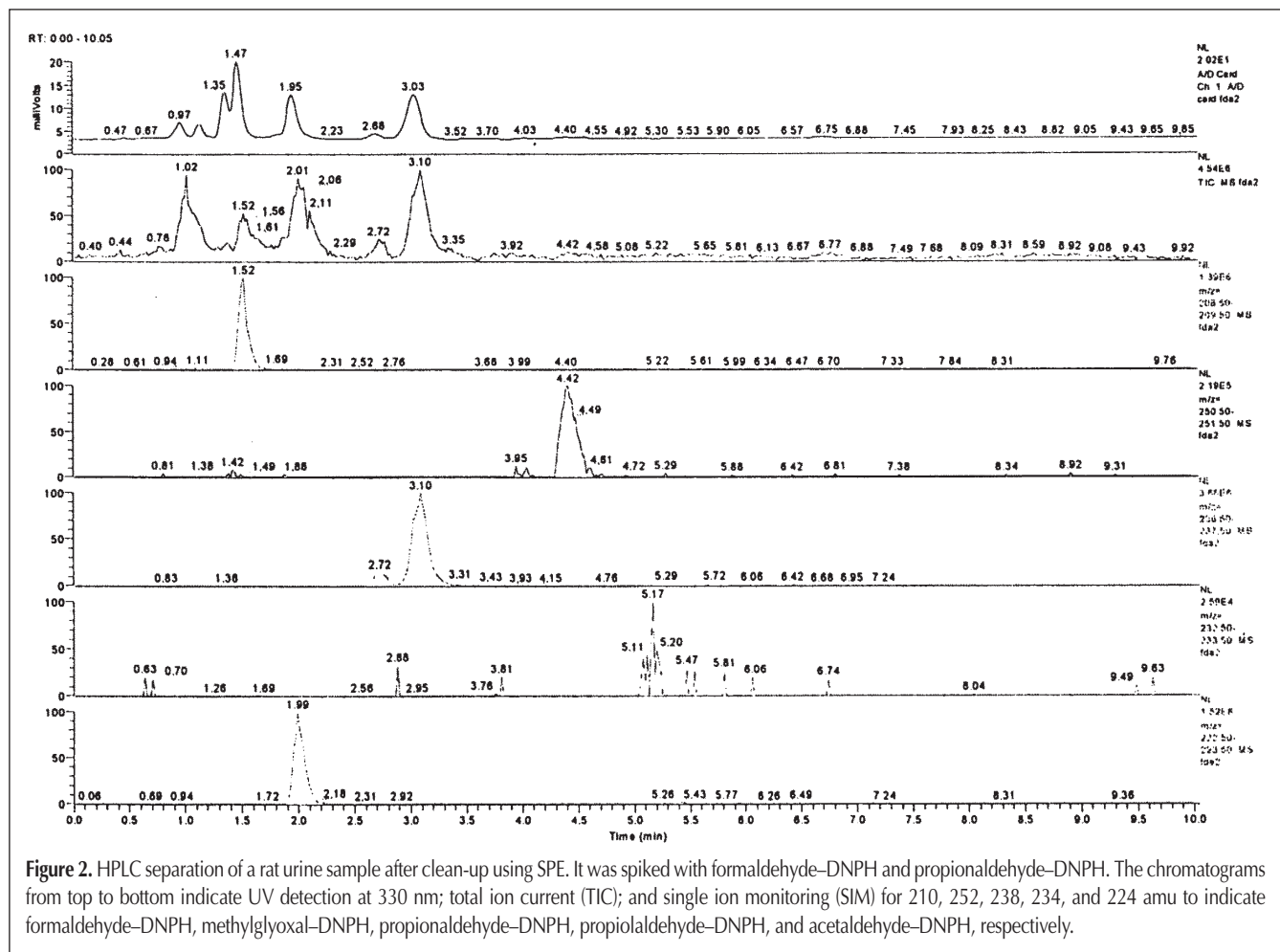
Figure 2. HPLC separation of a rat urine sample after clean-up using SPE. It was spiked with formaldehyde–DNPH and propionaldehyde–DNPH. The chromatograms from top to bottom indicate UV detection at 330 nm; total ion current (TIC); and single ion monitoring (SIM) for 210, 252, 238, 234, and 224 amu to indicate formaldehyde–DNPH, methylglyoxal–DNPH, propionaldehyde–DNPH, propiolaldehyde–DNPH, and acetaldehyde–DNPH, respectively.

Figure 3. Formaldehyde formation by the cytochrome P-450 cycle for an N-methylated drug. The *X* substituent can be either an alkyl group or a hydrogen.

Discussion

There are several quintessential steps that influence the fate and effects of drugs in the human and animal bodies. The individual steps are given by the so-called LADMER system (load, absorption, distribution, metabolism, elimination, and receptor effect).

The occurrence of *N*-oxides indicates an important place among the metabolites of several tertiary amine compounds (12–15). The *N*-oxide metabolites were identified and quantitated using both TLC and HPLC, and deprenyl-*N*-oxide was also identified. These results definitely suggest the dynamic participation of deprenyl-*N*-oxide in biotransformation through the cytochrome P-450 cycle. Formaldehyde is probably formed by oxygen migration from the nitrogen to the carbon of the *N*-methyl group. In addition to formaldehyde, deprenyl metabolism produces the other well-known metabolites, such as nordeprenyl, amphetamine, and methamphetamine (16).

Conclusion

The effects of the drugs depend on their presence at the target organ. The therapeutic level of (–)-deprenyl is approximately tenfold longer in the brain than that of the corresponding (+)-deprenyl (17). The metabolism often yields small molecular weight products. The small molecules can be formaldehyde, ammonia, hydrogen peroxide, etc. Two major metabolic procedures yield formaldehyde: the cytochrome P-450 dependent oxidation of drugs (Figure 3) and the semicarbazide-sensitive amine oxidase (SSAO) (18).

Quantitative determination of the formaldehyde level may be important when the effects of various alimentary habits, side effects of drugs, and drug–drug interactions are considered. Formaldehyde production can be essential from two different points of view. The excess of formaldehyde can cause various toxic symptoms, such as inflammation, etc. However, formaldehyde itself can be considered as a ubiquitous endogenous compound. The physiological level of formaldehyde may be beneficial in the counterbalancing of the oxidative stress. This procedure can be considered to be the reversal of the SSAO reaction. Therefore both the ammonia and the hydrogen peroxide are consumed by formaldehyde to produce methylamine, oxygen, and water as presented in the equation.

$$CH_3–NH_2 + O_2 + H_2O \xrightleftharpoons[SSAO]{SSAO} HCHO + H_2O_2 + NH_3 \qquad \text{Eq. 1}$$
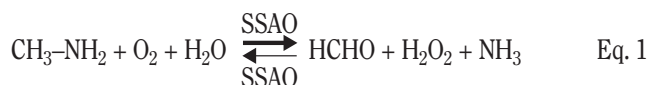
Formaldehyde determination is possible by either TLC or HPLC. To eliminate the interfering effect of environmental formaldehyde during the analysis, the use of radiolabelled formaldehyde producers are important. Thereby the results are reliable and permit a definite conclusion that the formaldehyde originated from the food component or from the drug in question. This was the case in our investigations, where the presence of radiolabelled formaldehyde in the rat's urine proved the formation of formaldehyde by oxidative demethylation of an N-methylated drug.

Monitoring the further fate of formaldehyde seems to be rather complicated. Either the use of the radiolabelled formaldehyde or employment of antibodies to both the formaldehyde–protein (19) or formaldehyde–DNA adducts may pave the way to trace the formaldehyde after its reaction. However, HPLC seems to remain the essential tool for the determination of free formaldehyde in the form of its 2,4-dinitrophenylhydrazone.

Acknowledgments

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