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Selected Ion Monitoring Analysis of Conjugated Metabolites of Methadone Using Biosynthetic Internal Standards for the Study of Methadone-Diazepam Interaction

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Abstract □ A methadone-diazepam interaction study in rats was performed in which conjugated metabolites of methadone were analyzed using deuterated biosynthetic internal standards. Diazepam (5mg/kg) was given to rats through a cannulated jugular vein and a subcutaneous dose of methadone (10mg/kg) was given. Bile was collected through the cannulated bile duct over a period of 24 hours. The deuterium label of the internal standards was found to be stable under conditions of the prolonged incubation. There was no significant difference in the excretion of the metabolites between the control and the diazepam treated rats. Feasibility of using biosynthetic internal standards with selected ion monitoring was established for the drug metabolism and kinetic studies.

Keywords □ Selected ion monitoring, Gas Chromatography-Mass spectrometry, Methadone-diazepam interaction, Analysis of conjugated metabolites, Biosynthetic internal standard, Stability of deuterium label, Ratio analysis.

Selected Ion Monitoring (SIM) of Gas Chromatography Mass Spectrometry(GCMS) has been widely used to quantitate drugs and their metabolites in biological fluids [1,2].

The method is known to provide high sensitivity in the analysis and ease in the work-up procedures especially when stable isotope labeled internal standards are used. This is owing to the fact that the stable isotope labeled internal standards have the same extractibility, stability, and the same capacity for the enzyme hydrolysis and derivatization as have the drug and metabolites to be analyzed.

In spite of the advantages of the SIM analysis, in practice, the method is frequently found to be not superior to the gas chromatography in terms of the sensitivity and economy of the analysis. For instance, methadone in plasma was analyzed,by SIM analysis, with a sensitivity limit of 5ng/ml either under electron impact ionization (EI)[3] or under chemical ionization (CI)[4], while the sensitivity of gas chromatography for the analysis of methadone and its major metabolite, 2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) in plasma and urine was of the order of 5-15ng/ml[5,6]. SIM analysis was also found to be a time consuming method compared with gas chromatography[7] since separate injections of the sample are required to analyze methadone and EDDP. Both methadone and EDDP could be analyzed by one injection of the sample using one internal standard with gas chromatography[5,6].

This paper is intended to describe a new advantageous application of SIM to the drug metabolism and kinetic studies employing internal standard (s) obtained *in vivo* from a deuterated precursor (biosynthetic internal standard). One

of such applications was demonstrated in this work by the analysis of conjugated metabolites of methadone.

It has been shown in various studies of metabolic interaction of methadone with other drugs that the interaction was reflected in the quantitative changes of conjugated metabolites. For instance, the biliary excretion of conjugated methadone metabolites was increased in rats by phenobarbital treatment[8] and the interaction between methadone and diazepam was shown in a decreased concentration of conjugated metabolites in urine[9].

In the previously described works[8,9], quantitative analysis of conjugated metabolites was achieved using radioisotopic method. The hydrolyzed metabolites were extracted quantitatively into organic solvents and chromatographed to separate each metabolites. In addition to the complications involved in the use of radioactive compounds, it was difficult to ensure complete separation of the metabolites. Depending upon the solvent system, marked variations in the amount of conjugated and nonconjugated metabolites of methadone were reported[8,10].

Therefore, the method of the use of biosynthetic internal standard for methadone-diazepam interaction study aimed at replacing radioisotopic method-thin layer chromatographic separation with SIM analysis employing biosynthetic internal standards. In stead of obtaining total radioactivity derived from water soluble metabolites without differentiating among each conjugated metabolites, the method can monitor ratios of each metabolites to deuterated biosynthetic metabolites added as internal standards.

EXPERIMENTAL METHODS

Materials

Methadone HCl and EMDP-$^2H_{10}$ (2-ethyl-5-methyl-3, 3-diphenyl-1-pyrroline-$^2H_{10}$) HCl were prepared as described[11]. Heparin (168 units/mg) was purchased from Sigma Chemical Co. Diazepam was obtained from Hoffman-La Roche, Montreal, Canada. Glucurase^R (β-glucuronidase, 5,000 sigma unit/ml, Sigma) was used for the hydrolysis of conjugated metabolites. Diazomethane was prepared by the method of Levitt[12]. Hexane, methylene chloride, and methanol distilled in glass were purchased from Caledon Laboratories Ltd., Ontario. Sodium acetate buffer (0.1M, pH 4.5), disodium citrate buffer (0.1 M, pH 2.0), and borate buffer (0.25M, pH 9.0) were used. Silastic (Dow Corning, 0.020 in. i.d., 0.037 in. o.d.), Polyethylene tubing-10 (Clay Adams, 0.011 in. i.d., 0.024 in. o.d.), and Polyethylene tubing-50 (Clay Adams, 0.023 in. i.d., 0.038 in. o.d.) were used for the animal surgery.

Analytical Method

Selected ion monitoring was performed using a Varian MAT 111 gas chromatograph-mass spectrometer. Accelerating voltage supply of the mass spectrometer was modified to permit scanning the accelerating voltage using a Varian 620L computer. The mass spectrometer was operated with an electron ionization voltage of 70 eV and a source temprature of 285°C. Trap current was 300 uA. A glass column (1.6m×2mm i.d.) packed with 3% OV-17 on 80/100 mesh Chromosorb W (HP) was used with carrier gas (He) at 20 ml/min. The operating conditions were as follows; column temperature, 200°C programmed to 280°C at 10°C/min, separator and inlet line temperature, 290°C, and injector temperature, 220°C.

Monitoring ions for the SIM analysis of the conjugated HOEMDP and conjugated DiHO-EMDP were chosen from base peak ions of CH_3OEMDP, CH_3OEMDP-$^2H_9$, $(CH_3O)_2EMDP$,

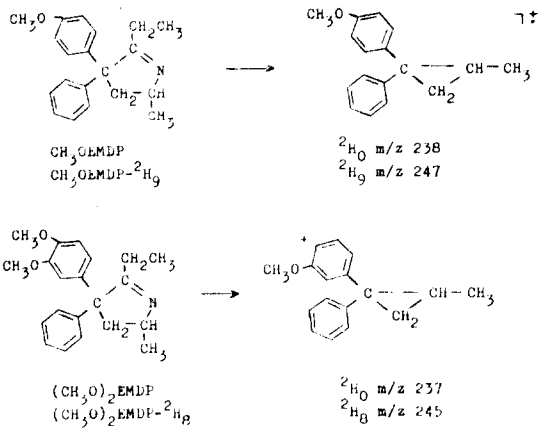

Fig. 1: Base peak fragment ions for CH_3OEMDP, CH_3OEMDP-$^2H_9$, $(CH_3O)_2EMDP$, and $(CH_3O)_2EMDP$-$^2H_8$

and $(CH_3O)_2EMDP$-$^2H_8$ as shown in Fig. 1. The mass spectra were obtained from the total ion current (TIC) profile of the extract of the conjugated fraction isolated from EMDP and EMDP-$^2H_{10}$-dosed rat bile[13].

Treatment of Animals

Male Wistar rats (200~300gm) obtained from Canadian Bio-Breeding Farm Laboratories (Montreal, Quebec) were maintained on a standard diet of Purina Lab Chow (Ralston Purina Co. of Canada) and water *ad libitum*. The rat was jugular vein cannulated by the method of Upton[14]. The external jugular vein was exposed by tissue forceps and a 3cm piece of Silastic tubing was connected to a 3 cm PE-50 by means of a piece of 22 gauge hypodermic needle. After the jugular vein cannulation, bile duct cannulation was performed as described by Lambert[15]. PE-10 tubing was inserted into the common bile duct. The total time spent for the jugular vein and bile duct cannulation was 20-30 min. After the bile duct cannulation, diazepam (5mg/kg in 2 ml vehicle) or vehicle only was given through the jugular vein at an infusion rate of 2 ml/hour. The diazepam was dissolved in a solution of propylene glycol (40%), ethanol (10%), benzyl alcohol (1.5%), and sodium benzoate (5%) in water. During the infusion, the surgical area of the bile duct cannulation was sutured. After the infusion, the PE-50 tubing was cut short and sealed. The animal was resutured with the tubing inside the layer of the skin. Shortly before methadone dosing, one hour after the start of the infusion, the rat was placed in a restraining cage and methadone HCl (10mg/kg in 2 ml saline) was given s.c.. Bile was collected from the cannulated bile duct in preweighed scintillation vials at different times after the methadone (1, 2, 5, 11, and 23 hours). The bile collected before the methadone dose served as a blank.

Sample Preparation Procedures

The conjugated internal standards were prepared by dosing to 3 rats with 20mg/kg EMDP-$^2H_{10}$ s.c.. Bile was collected for the period of 24 hours. The bile was extracted once with methylene chloride to remove EMDP-$^2H_{10}$. The remaining bile was diluted to 100 ml with water and frozen in an Erlenmeyer flask at −20°C until used. This solution was used directly as internal standards to measure conjugated metabolites.

A bile sample (0.2~0.4 ml) was mixed with 1 ml of a conjugated internal standard solution. The mixture (pH 9.0~9.5) was extracted with methylene chloride (15ml). The aqueous layer was freeze dried and 1.5 ml of buffer solution (pH 4.5) was added (final pH 5.0). After the mixture was incubated with Glucurase (0.2 ml) for 24 hours at 37°C, the pH of the solution was adjusted with 2 ml of borate buffer (pH 9.0) to pH 8.5~9.0. The mixture was extracted with methylene chloride (15ml). The methylene chloride extract was dried over anhydrous Na_2SO_4 and taken to dryness. The dried

residue was dissolved in 1ml of methanol and treated with diazomethane. The sustained yellow colored methanol solution was evaporated and the pH was adjusted with 1.5ml of pH 2.0 buffer. The mixture was vortex mixed with 5 ml of hexane to remove endogenous materials. The pH of the aqueous part was adjusted to be alkaline with 1 N NaOH (0.5ml) and extracted with methylene chloride (10ml). The methylene chloride solution was dried over Na_2SO_4 and taken to dryness under N_2. The final residue obtained was dissolved in suitable volume of methanol for SIM analysis. Ions at m/z 247, 246, 238, and 237 were monitored for CH_3OEMDP and m/z 245, 244, 237 and 236 for DiCH_3OEMDP. The intensity ratios, m/z 238/m/z247 and m/z 237/m/z245 were selected for the analysis of HOEMDP and DiHOEMDP, respectively.

Table I: Stability of deuterium in HOEMDP-$^2H_9$.

Time(h)	246/247	238/247	237/247
12	0.435	−0.0062	0.0005
24	0.439	0.0060	0.0062
48	0.432	0.0054	0.0041
96	0.441	−0.0070	−0.0001
Time(h)	246/247	245/247	244/247
12	0.446	0.359	0.118
24	0.437	0.403	0.113
48	0.461	0.407	0.113
96	0.437	0.407	0.125

Samples were monitored twice with the first set of ions, m/z 247, 246, 238, 237 and the second, m/z 247, 246, 245, 244. The stability of the label, the presence of impurities and the stability of the ion focussing were examined using the intensity ratios of m/z 246 to m/z 247. The ion, m/z 238 was chosen for CH_3OEMDP and m/z 247 for CH_3OEMDP-$^2H_9$. The values were the average from two incubations at each incubation time. Negative values resulted from background subtraction.

Stability Experiments

The conjugated internal standard (2ml) was incubated with 0.5ml of Glucurase for 12, 24, 48, and 96 hours with subsequent extraction using the same procedures as described for the bile samples. Specific ions, m/z 247, 246, 245, 244 and m/z 247, 246, 238, 237 for CH_3OEMDP-$^2H_9$ and m/z 247, 246, 245, 244 and m/z 245, 244, 237, 236 for $(CH_3O)_2$EMDP-$^2H_8$ were monitored which would indicate stability of the deuterium label. Details are given in Table I and Table II.

Table II: Stability of deuterium in DiHOEMDP-$^2H_8$.

Time(h)	244/245	237/245	236/245
12	0.363	0.0088	0.0091
24	0.359	0.0069	0.0052
48	0.364	0.0059	0.0060
96	0.369	0.0066	0.0077
Time(h)	247/245	246/245	244/245
12	0.0296	0.215	0.359
24	0.0341	0.211	0.356
48	0.0266	0.214	0.359
96	0.0250	0.207	0.359

Samples were monitored twice with the first set of ions, m/z 245, 244, 237, 236 and the second, m/z 247, 246, 245, 244. The stability of the label, the presence of impurities, and the stability of the ion focussing were examined using the intensity ratios of m/z 244 to m/z 245. The ion, m/z 237 was chosen for DiCH_3OEMDP and m/z 245 for DiCH_3OEMDP-$^2H_8$. The values were the average from two incubations at each incubation time.

RESULTS AND DISCUSSION

Stability Studies

An absence of changes in ion intensity ratios of the conjugated metabolites following 96 hours of incubation time was indicative of the stability

of the deuterium label (Table 1, II). Even in the presence of an activating group such as the hydroxyl group on the ring, the deuterium was stable. This result is consistent with that of a report that the deuterium label on the ring of *p*-hydroxyephedrine obtained by metabolic processes was found to be stable during the metabolism and the subsequent enzyme hydrolysis of the conjugate for the analysis[16].

The stability studies of conjugated metabolites also showed that the ion intensity ratios, m/z 238/247, m/z 237/247. m/z 237/245, and m/z 236/245 were less than 1%. This implies that there was an absence of impurities in endogenous bile and no contribution from labeled metabolites which might interfere in the analysis of unlabeled metabolites. The potential overestimation of methadone conjugated metabolites arising from analytical interferences such as endogenous materials and conjugated metabolites of diazepam was also examined. If there is no interference, then monitoring of consecutive ions should give constant ratios. The ions monitored were m/z 246/247 for CH_3OEMDP-$^2H_9$ and m/z 241/245 for $DiCH_3OEMDP$-$^2H_8$. A sudden change of the intensity ratios indicated in most cases disruption of the ion focussing arising from an unstable magnetic field. The fact that blank samples collected for the first one hour after the diazepam dose did not interfere with the ion intensity ratios indicated an absence of any interference from diazepam metabolites. It has been reported that during the first one hour, 45% of the radioactivity injected in the form of (5-^{14}C)-diazepam into the rat appeared in the bile[17].

Independent of the set of the ions monitored (m/z 247, 246, 245, 244 and m/z 247, 246, 238, 237), the intensity ratios of m/z 246 to m/z 247 monitored for CH_3OEMDP-$^2H_9$ were not deviated significantly. Consective ion monitoring (m/z 247, 246, 245, 244) and monitoring four separate ions (m/z 245, 244, 237, 236) also did not give significant deviations in the

Table III: Effect of diazepam treatment on the bile flow of rats.

Time(h)	1-M	1-DM	2-M	2-DM	3-M	3-DM	4-M	4-DM
0- 2	3.85	4.04	4.28	4.07	3.52	4.41	3.72	5.22
2-11	26.00	28.08	13.81	20.89	23.18	21.92	19.37	26.79
11-23	29.90	27.48	23.72	17.69	25.17	25.66	17.97	18.85
Total	59.75	59.60	41.81	42.65	51.87	51.99	41.06	50.86

Time(h)	M	DM	p
0- 2	3.842±0.321	4.435±0.549	>0.10
2-11	20.590±5.273	24.420±3.546	>0.20
11-23	24.190±4.915	22.420±4.872	>0.20
Total	48.622±8.906	51.275±6.937	>0.20

Paired experiments were performed using four rats for methadone treatment (M) and another four rats for methadone and diazepam treatment (DM). The values denote the amount of bile (g/kg rat) collected over the time periods, 0-2, 2-11, and 11-23 hours following the methadone dose. The values in the lower part of the Table represent mean±SD obtained for each of the two groups. The value p was calculated by using Student's t test.

intensity ratios of m/z 244 to m/z 245 monitored for $DiCH_3OEMDP-^2H_8$. These implied good precision of the analytical system.

Bile Collection

Table III describes the bile volumes collected from each rat for the methadone-diazepam interaction study. The total amount of collected bile averaged 50ml/kg for 23 hours which was a little lower than the bile flow (60-100ml/kg/24 hours) described by Johnson and Rising[18]. Taking it into account that the fluid replacement by infusion was not carried out, the bile flow appeared adequate to obtain reliable data for the drug interaction study.

Diazepam did not influence bile flow during the 23 hour time period of the study ($p>0.20$). A similar result was reported by El-Hawari and Plaa[19] in their studies of the effects of diazepam on the biliary excretion of diphenylhydantoin.

Analysis of Conjugated Metabolites

SIM chromatograms of mono-and dihydroxy EMDP isolated from rat bile are shown in Fig. 2. The ions chosen to monitor were the base peaks of the compounds and had high selectivity.

Diazomethane treatment and selective extraction of the diazomethane treated samples provided several advantages in the analysis. Underivatized dihydroxy EMDP was not detectable in the TIC profile of the conjugated fraction. Diazomethane treatment and hexane extraction of the sample permitted complete removal of any possible interferences. Derivatization by diazomethane methylation also had advantages in terms of preventing oxidation of the phenolic and catechol type metabolites.

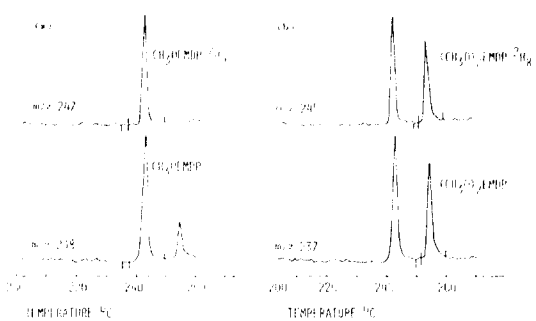

Fig. 2: SIM chromatograms for (a) HOEMDP and (b) DiHOEMDP analysis.

Calibration curves were prepared by using one of the bile samples in different volumes (Table IV). The ratios obtained from natural sample analysis were within the calibration range. The intensity ratios of m/z 246 to m/z 247 and those of m/z 244 tom /z 245 were 0.43~0.46 and 0.36 ~0.37, respectively, indicating absence of interference in the analysis i.e. the values were the same as those shown in Tables I and II.

As shown in Tables V and VI an increased excretion of conjugated metabolites was found during the first 2 hours in bile excretion of the methadone-diazepam treated rats. For HOEMDP 5.7% and 16.8% were excreted during the first 2 hours in the control and the diazepam treated rats, respectively. In the case of DiHOEMDP, the percentages excreted were 5.3% and 10.7%

Table IV: Calibration curve data for conjugated metabolites.

Sample(ml)	HOEMDP analysis		DiHOEMDP analysis	
	246/247	238/247	244/245	237/245
1.6	0.443	1.440	0.373	1.204
0.8	0.431	0.712	0.365	0.619
0.4	0.461	0.359	0.366	0.341
0.2	0.448	0.162	0.367	0.153
0.1	0.466	0.092	0.377	0.074
Slope	0.903		0.748	
Intercept	−0.00683		0.014	
r^2	0.9998		0.9992	

1ml of internal standard(as prepared in Experimental) was added. The values denote intensity ratios of the ions with average of two determinations.

Table V: Excretion of HOEMDP from methadone and methadone-diazepam treated rats.

Time(h)	1-M	1-DM	2-M	2-DM	3-M	3-DM	4-M	4-DM
0- 2	0.447	3.654	0.961	3.275	2.893	5.092	0.874	4.316
2-11	16.545	18.888	10.377	11.650	20.609	15.400	12.647	16.200
11-23	6.270	2.966	9.827	5.469	2.848	4.554	5.995	5.454
Total	23.262	25.508	21.165	20.394	26.350	25.046	19.516	25.970

Time(h)	M	DM	p
0- 2	1.294±1.089	4.084±0.797	<0.01
2-11	15.044±4.499	15.534±2.988	>0.20
11-23	6.235±2.853	4.611±1.177	>0.20
Total	22.573±2.948	24.229±2.584	>0.20

The values denote intensity ratios(ratio/kg rat) of m/z 238 to m/z 247 with average of two determinations. Monitoring ions were m/z 247, 246, 238, and 237. Other details are in Table III.

Table VI: Excretion of DiHOEMDP from methadone and methadone-diazepam treated rats.

Time(h)	1-M	1-DM	2-M	2-DM	3-M	3-DM	4-M	4-DM
0- 2	1.794	3.302	0.509	2.009	1.840	2.218	0.726	2.028
2-11	16.510	14.968	10.537	14.160	18.270	14.376	13.511	16.671
11-23	7.079	2.376	11.171	7.526	2.122	5.150	7.243	3.711
Total	25.383	20.646	22.217	23.695	22.232	21.744	21.480	22.410

Time(h)	M	DM	p
0- 2	1.217±0.698	2.389±0.615	<0.05
0-11	14.707±3.404	15.043±1.137	>0.20
11-23	6.903±3.706	4.690±2.203	>0.20
Total	22.827±1.739	22.122±1.275	>0.20

The values denote intensity ratios (ratio/kg rat) of m/z 237 to m/z 245 with average of two determinations. Monitoring ions were m/z 245, 244, 237 and 236. Other details are in Table III.

for the control and the diazepam treated rats, respectively. However, when the excretion of conjugated metabolites was followed over the entire 23 hour period, no difference was observed between the two groups (HOEMDP $p>0.20$, DiHOEMDP $p>0.20$).

The result was quite different from that reported by Liu et al.[9], in that water soluble metabolites of methadone in urine and liver was significantly decreased by diazepam administration, but was consistent with a report of Shannon et al.[20] whose experiment done with 10mg/kg i.p. diazepam and 0.6mg/kg i.p. methadone failed to show any interaction in mice when plasma and brain levels of methadone were measured. A recent paper also showed lack of effect of diazepam on methadone metabolism in methadone-maintained addicts where the effects

and kinetics of methadone and its major pyrrolidine metabolite were studied[21].

The reason for the transient increase of the metabolites in the initial 2 hour period was not sought but could be due to competition of diazepam with methadone for the plasma protein binding. An effect of diazepam on increasing free drug levels was indicated in studies of diazepam-diphenydantoin[19]. But the effect by an increase of free drug might be minimal because in the case of the drug which has high hepatic extraction, drug binding to the plasma is not a critical factor in the hepatic metabolism[22].

In conclusion, the concomitant administration of diazepam with methadone in rats did not affect biliary excretion of the conjugated metabolites. Diazepam did not interact with methadone at the hepatic metabolism level of the formation of conjugated metabolites nor on the transport of the metabolites by the biliary excretion route. This result is not consistent with that by Liu et al.[9] in which the change of water soluble metabolite level in urine and liver was considered to be an indicator of the methadone-diazepam interaction.

Potential Use of Biosynthetic Internal Standards with SIM for the Drug Metabolism and Kinetic Studies

The isotope ratio of the mixture measured by monitoring selected ions can be used as a direct measure of the mole ratio in the mixture where overlapping ions are not present between the sample and the internal standard[23].

$$aX/Y=Rm$$

where a is the constant, X/Y is the mole ratio of sample to labeled internal standard in the mixture, and Rm is the isotope ratio of the mixture. a/Y is obtained from standard ratio and the amount of added internal standard. Standard ratio is an isotope ratio of the equal amount of sample to internal standard[7]. Even when a/Y value is not known, the ratio of unlabeled to labeled (Rm) can be used to measure the change of drug concentration (X) because a/Y value can be kept constant.

Besides the application of this ratio analysis method employing biosynthetic internal standards to the methadone-diazepam interaction study where authentic conjugated metabolites are not available, the method will be effectively used when induction of a few specific metabolic pathways by an agent is examined. In this case, internal standards for the corresponding metabolites can be prepared from one stable isotope labeled precursor. More specifically, this method could be applicable to the biphenyl metabolism studies published by Benford et al.[24] and Halpaap-Wood et al.[25].

In addition, applicability of the methodology to the pharmacokinetics can be demonstrated with an equation of two compartment model[26].

$$Rm=aX/Y=aXo/VcY \left[\frac{(\alpha-k_{21})}{\alpha-\beta} e^{-\alpha t} + \frac{(k_{21}-\beta)}{\alpha-\beta} e^{-\beta t} \right]$$

All kinetic constants, α, β, k_{21}, aXo/VcY are calculable with ratios (Rm) at times (t) by the NONLIN[27].

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