FORENSIC SCIENCE JOURNAL SINCE 2002

Forensic Science Journal
2011;10(1):39-47
Available online at:fsjournal.cpu.edu.tw

Quantitative and enantiomeric compositions of methamphetamine and amphetamine in rat tissues following the administration of benzphetamine

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Received: Sep 30, 2011 / Accepted: Oct 21 , 2011

Abstract

Benzphetamine is known to metabolize to methamphetamine and amphetamine in biological systems. An animal study was conducted to understand the distribution of these metabolites in various tissues. Rats were divided into control (n = 6), 2-hour (n = 6), and 4-hour (n = 6) groups. The latter two groups were administered (IV) 30 mg/Kg benzphetamine and scarified 2 and 4 hours after drug administration. A relatively simple analytical scheme, including liquid-liquid extraction, asymmetric chemical derivatization (*l*-N-trifluoroacetyl-*l*-prolyl chloride or l-TPC), and GC-MS analysis, was developed for simultaneous determination of methamphetamine and amphetamine in kidney, liver, brain, heart, and serum. Calibration curves (50–1000 ng/g) established for methamphetamine and amphetamine in various tissues exhibit good linearity (correlation coefficient >0.999). Major metabolites of benzphetamine are *d*-enantiomers and the concentration of *d*-amphetamine was found higher than *d*-methamphetamine in all tissues, indicating *N*-demethylation as a major route of the metabolism. The concentrations of the metabolites in various tissues are in the order of kidney > brain ~ liver > heart ≥ serum. These metabolites' concentrations increase during the first two hours and start to descend between two to four hours after dose.

Keywords: Benzphetamine, enantiomeric analysis, *N*-demethylation

Introduction

Having been known to be a precursor compound of methamphetamine (MA) and amphetamine (AP) in biological system for almost 40 years [1], benzphetamine (BZ) has been subjected to a number of studies with emphasis in differentiating the ingestion of BZ from MA [1–9]. These studies have well established that (a) BZ is quickly converted to the metabolites, with low or undetectable parent compound found in urine; (b) the metabolites (MA and AP) are in d-forms; and (c) the concentration of AP is typically higher than that of MA suggesting *N*-demethylation as a major route of metabolism (Fig. 1) [8].

With these information in mind, this animal study was conducted to better understand the distributions of the metabolites in various tissues.

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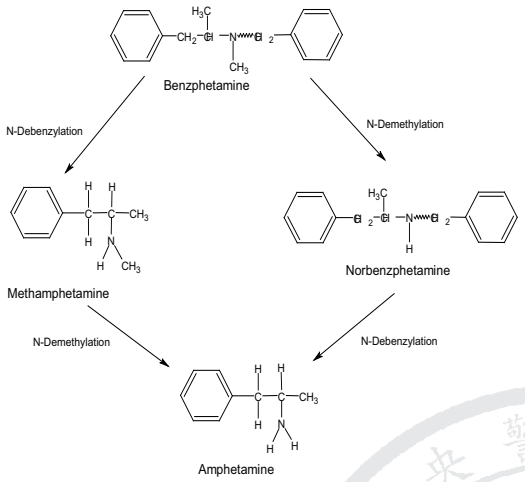

Fig. 1. Metabolic pathways of benzphetamine[8].

Experimental

Materials

Standards and derivatization reagents were purchased from the following sources: BZ, Sigma-Aldrich (St. Louis, MO); *d,l*-MA and *d,l*-AP (methanolic solution, 1 mg/mL) and *d,l*-MA-d_8 and *d,l*-AP-d_{10} (methanolic solution, 100 µg/mL), Cerilliant Co. (Austin, TX); and (S)-(–)-N-(trifluoroacetyl)-prolyl chloride (*l*-TPC), Aldrich Chemical Co. (St Louis, MO).

Animal experiment

Experiments related to animal studies were conducted following the protocols established by Taiwan University College of Medicine. Female Wistar rats were maintained at 24 °C, with light for 12 hours/day, and fasted for 24 hours. They were divided into three groups (n = 6 for each group) before saline or drug administrations.

The control group was administered (IV) with 0.9% normal saline, while the 2-hour and the 4-hour groups were administered (IV) with 30 mg/kg BZ and sacrified at 2 and 4 hours, respectively, after drug administration.

Sample preparation, liquid-liquid extraction and l-TPC-derivatization

Whole blood was set for agglutination, centrifuged, and the serum on the top was collected. Organs were first washed with icy saline, dried, weighed, then added 3× saline (w/w) and homogenized in a Polytron Homogenizer (GMBH-Kriens, Switzerland).

To each of the 2-mL fortified or case rat organ specimen in a screw-cap test tube was added 0.5 mL saturated K_2CO_3 solution, 50 µL *l*-TPC and 25 µL IS solution (250 ng/mL in the test sample). The mixture was shaken for 10 min, then centrifuged for 5 min at 5000 rpm. The upper layer was transferred to another screw-cap test tube and dried under N_2 at 50 °C. The residue was reconstituted with 200 µL hexane for GC-MS analysis [10-17].

GC-MS analysis

The following instrumentation and protocols were adapted for GC-MS analysis. A HP-6890 gas chromatograph was equipped with a HP-5 MS capillary column (30 m × 0.2 mm I.D., 0.33 µm film thickness) and coupled to a HP-5973 series mass selective detector (MSD). Temperature settings for the injector and the interface were 250 and 280 °C, respectively. The oven was programmed from 150 to 250 °C at 20 °C/min, with 5-min holds at the initial and the final temperatures.

For quantitative analysis, the MSD was operated in selected ions monitoring (SIM) mode with *m/z* 91, 118, 237; 97, 128, 241 and 58, 176, 251; 65, 184, 258 designating AP and MA and their respective ISs. Data derived from ions underlined were adapted for quantitative determination.

Statistical analysis

Data collected from each animal and tissue vary substantially and were statistically analyzed using SPSS 10.0 through Microsoft Excel 2002 data input. One-way ANOVA were performed to determine whether concentration differences of MA or AP in various tissues were significant [18,19]. With two metabolites (MA and AP) of benzphetamine and two metabolism periods (2-hour and 4-hour) as the parameters, the one-way ANOVA

was performed four times. Since multiple comparisons were involved in the process, Bonferroni adjustment [20,21] was applied to the resulting parameters. Multivariate analysis of variance [22] was also performed to determine whether the differences in the concentrations between MA and AP and between 2-hour and 4-hour in various tissues were significant. Three steps were involved in the process. First, correlation coefficients derived from Spearman correlation [23] were evaluated to determine whether metabolite concentrations were indeed related to drug type and metabolism period. Two-way ANOVA were then performed [18,19], followed by applying, whenever appropriate, Bonferroni adjustment of the resulting parameters [20,21].

Results and discussion

Calibration curve, LOD, LOQ

Analytical data derived from standards, prepared by spiking 35, 40, 45, 50, 100, 250, 500, and 1000 ng/g (ng/mL) of AP and MA (in *d*- and *l*-forms) into various homogenized blank tissues. The qualifier, quantifier ions and retention time of method were shown in Table 1. All produced correlation coefficients >0.999, indicating linear calibration within the 45–1000 ng/g (ng/mL) concentration range in Table 2.

The method limit of quantitation (LOQ) was determined by a definition currently prevailing in the forensic practice [10,24]. After serial total-analyses of blank tissues spikes containing lower and lower concentrations of the relevant analytes (i.e., 1000, 500, 250, 100, 50, 45, 40, 35 ng/mL, respectively, each of *d*-MA, *l*-MA, *d*-AP, and *l*-AP), the respective lowest concentrations of the analytes that analyzed accurately within±20% of the respective target concentrations were designated as the respective LOQs of the analytes, the string being that the two qualifier-ion-abundance ratios of each derivatized AP or MA matched within±20% of those of the calibrators. Due to practical feasibility, the method limit of detection(LOD) was, however, simply defined as the lowest concentration of the analyte that gives the two qualifier-ion-abundance ratios matching within ±20% of those of the calibrators.

Following the same protocol, test specimens were analyzed and the resulting concentrations of MA and AP are shown in Table 3. Mean concentrations of MA and AP derived from various tissues are further presented in Fig. 2.

Table 1 Qualifier and quantifier ions selected for the four diastereomeric pairs of TP-amphetamines

***l*-TPC derivatized analyte**	**Qualifier ions (*m*/*z*)**	**Quantifier ions (*m*/*z*)**	**Retention time**
l-TP-AP	237 118 91	237	11.082
d-TP-AP	237 118 91	237	11.232
l-TP-AP-d10	241 128 97	241	11.037
d-TP-AP-d10	241 128 97	241	11.187
l-TP-MA	251 176 58	251	12.403
d-TP-MA	251 176 58	251	12.573
l-TP-MA-d8	258 184 65	258	12.346
d-TP-MA-d8	258 184 65	258	12.513

Table 2 Calibration equations, linearity ranges, linearity (r^2) of metabolites in tissues

Tssues	Analyte	Calibration equation	Linearity ranges (ng/mL)	Linear ity (r^2)	LOD (ng/mL)	LOQ (ng/mL)
Heart	*l*-AP	$y = 0.0048x - 0.0243$	45-1000	0.9994	40	45
	d-AP	$y = 0.0047x - 0.0213$	45-1000	0.9993	40	45
	l-MA	$y = 0.0043x - 0.0063$	45-1000	0.9996	40	45
	d-MA	$y = 0.0044x - 0.0123$	45-1000	0.9997	40	45
Liver	*l*-AP	$y = 0.0043x + 0.0192$	45-1000	0.9999	40	45
	d-AP	$y = 0.0046x - 0.0182$	45-1000	0.9999	40	45
	l-MA	$y = 0.0042x - 0.0008$	45-1000	0.9999	40	45
	d-MA	$y = 0.0042x - 0.0372$	45-1000	0.9999	40	45
Brain	*l*-AP	$y = 0.0040\ x + 0.0731$	45-1000	0.9996	40	45
	d-AP	$y = 0.0045x + 0.0143$	45-1000	1	40	45
	l-MA	$y = 0.0040x + 0.0731$	45-1000	0.9996	40	45
	d-MA	$y = 0.0041x + 0.0239$	45-1000	0.9998	40	45
Serum	*l*-AP	$y = 0.0043x + 0.0655$	45-1000	0.9993	40	45
	d-AP	$y = 0.0043x + 0.0597$	45-1000	0.9992	40	45
	l-MA	$y = 0.0039x + 0.0543$	45-1000	0.9993	40	45
	d-MA	$y = 0.0040x + 0.046$	45-1000	0.9995	40	45
Kidney	*l*-AP	$y = 0.0047x - 0.0193$	45-1000	0.9994	40	45
	d-AP	$y = 0.0047x - 0.0157$	45-1000	0.9997	40	45
	l-MA	$y = 0.0043x - 0.0055$	45-1000	0.9995	40	45
	d-MA	$y = 0.0043x - 0.0107$	45-1000	0.9996	40	45

Concentrations of MA and AP in various tissues in two and four hours after drug administration

Relationship of benzphetamine metabolites in tissues within three groups was measured by Spearman correlation test, and one-way ANOVA was performed to compare the association between two metabolism period and different tissues, and the result was shown in Table 4. With substantial differences among the numerical data resulting from different experimental subjects, it is not certain whether the observed differences for some data were indeed significant. Thus, multivariate analysis of variance was performed and the statistical parameters are listed in Table 5.

In this study, 18 rats were divided into three groups randomly: control group (n=6), 2-hours (n=6) and 4-hours (n=6). Concentrations of MA and AP were detected in various tissues, and distribution was shown in Table 3 and Fig. 2. In Table 3, AP-concentration was higher than MA-concentration in almost tissues in 2-hours and 4-hours, the concentration of MA and AP in tissues within 2-hour were higher than 4-hour. Comparing these two time period, metabolite concentration increased from 0-hour to 2-hour and decreased from 2-hour to 4-hour, and here has the same trend in Fig. 2.

Table 3 Concentrations distribution (ng/g or ng/mL) of methamphetamine (MA) and amphetamine (AP) in subjects' tissues.

	Brain		Heart		Kidney		Liver		Serum	
Specimen	MA	AP	MA	AP	MA	AP	MA	AP	MA	AP
2H[a]										
2H-1	225	674.5	85.83	162.3	913.1	1905	277.3	666.6	20.75	55.23
2H-2	277.8	512.4	99.91	167.5	722.5	1220	409.8	575.5	13.08	23.51
2H-3	397.4	553.4	105.7	117.1	1462	1896	427.3	584.7	25.71	45.48
2H-4	345.4	671.6	98.48	211.2	944.6	1604	406.8	662.9	24.13	58.61
2H-5	568.5	1275	136.4	245.3	1622	3002	556.3	1310	36.64	112.4
2H-6	331.2	727.6	159.1	224.7	470.9	1297	558.1	1113	59.63	81.92
Mean	360.9	735.8	114.2	188	1023	1821	439.3	818.7	29.9	62.8
Std dev	46.9	113	11.3	19.4	179	264	43.2	128	6.7	13
4H[b]										
4H-1	282.4	201.8	52.35	54.32	308.7	463.1	177	258.7	nd	7.778
4H-2	253.8	573.2	144.9	227.6	504.6	1163	317.8	663.6	17.73	59.3
4H-3	188.2	457.8	94.26	119.1	623.8	1286	210	433.3	21.65	36.09
4H-4	288.9	253.6	35.6	67.72	419.3	658.8	261.6	319.5	20.78	7.099
4H-5	312.1	291.9	95.25	92.72	313.8	437.3	222.8	282	19.35	18.89
4H-6	161.9	800.9	99.54	177	1526	2598	260.5	580.2	22.77	25.13
Mean	247.9	429.8	87	123.1	616	1101	241.6	422.9	20.8	29.9
Std dev	24.5	93.3	15.8	27.4	188	333	20.1	43.2	5.9	9

a: Metabolites concentrations of each rat in 2 hours.

b: Metabolites concentrations of each rat in 4 hours

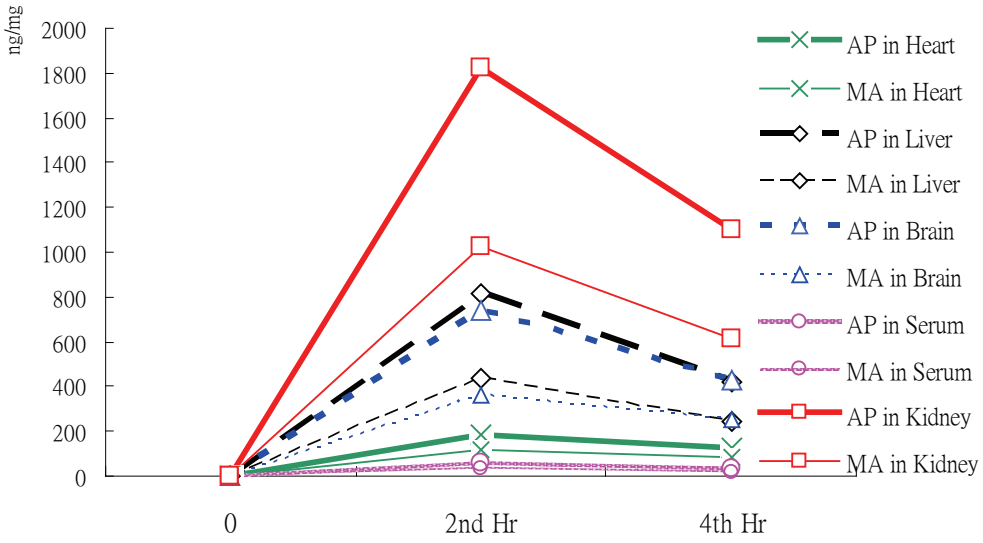

Fig. 2 Concentrations of methamphetamine and amphetamine in various tissues at 2 and 4 hours after administration(IP) of benzphetamine

Concentrations variation of MA and AP in tissues during metabolism time

In Spearman correlation testing, there's no difference within subject health status. After understanding the subjects have the same health statement in this study, than used one-way ANOVA and multivariate analysis of variance to explore metabolic mechanism in different tissues and metabolism time.

In one way ANOVA, the concentration variance in tissues of two metabolites (MA and AP) was calculated separately (Table 4). AP and MA concentration of 6 tissues were both different significantly in 2-Hour and 4-Hour (p value<0.05), average concentration of kidney in AP and MA were higher than other organs.

The multivariate analysis of variance was used to investigate differences between factors which related in metabolites concentrations under adjusted interaction effect. Based on the F-values shown in Table 5, differences in the concentrations of MA and AP and the concentrations variation of MA and AP in 6 tissues and 2 metabolism period (2 and 4 hours after drug administration) were all significant(p value all less than 0.01). While differences in the concentrations of MA and AP in various tissues are generally significant and the levels of these metabolites in these tissues are in the order of kidney > brain ~ liver > heart >serum, p-values shown in Table 5. indicate the differences of these metabolites' concentrations in brain and liver are clearly not significant, while the differences of their concentrations between heart and serum are not as significant as others.

Enantiomeric form and relative concentrations of MA and AP

Using *l*-TPC as the derivatization reagent, analytical data indicate both MA and AP observed are clearly d-enantiomers. With two exceptions (serum specimens from subjects 4H-4 and 4H-5), the concentrations of AP in all tissues, collected at 2 and 4 hours after drug administration, are higher than that of MA. This is different from what have been reported for urine specimens collected from the single subject studied by Budd and Jain [4] and two (out of 10) subjects studied by Cody and Valtier [8]. Regardless, with the metabolism rate of MA to AP is unlikely to exceed 20% [19], data derived from this current study further support *N*-demethylation of BZ as a competitive metabolic route.

Table 4 One way ANOVA test for metabolite in different time of each tissue separately

Metabolite				Mean ± SE	F test[a]	p value[b]
AP[c]	**Time[e]**	**2hrs[f]**			24.4	**<0.0001**
			Heart	188.0±19.4		
			Liver	818.7±127.7		
			Brain	735.8±112.9		
			Serum	62.8±12.6		
			Kidney	1820.7±263.9		
		4hrs[f]			7	**0.001**
			Heart	123.1±27.4		
			Liver	422.9±69.0		
			Brain	429.8±93.3		
			Serum	29.9±9.0		
			Kidney	1101.0±332.8		
MA[d]	**Time[e]**	**2hrs[f]**			20.9	**<0.0001**
			Heart	114.2±11.3		
			Liver	439.3±43.2		
			Brain	360.9±46.9		
			Serum	29.9±6.7		
			Kidney	1022.5±179.3		
		4hrs[f]			7.2	**<0.0001**
			Heart	87.0±15.8		
			Liver	241.6±20.1		
			Brain	247.9±24.5		
			Serum	20.8±5.9		
			Kidney	616.0±188.4		

a: One-Way ANOVA test value.

b: Significant level was 0.05 in this study.

c: AP was one of metabolites of Benzphetamine.

d: MA was one of metabolites of Benzphetamine.

e: There were two time period mechanism (2-hours and 4-hours)after drug administration be explored in this study.

f: hrs=hours.

Table 5 Multivariate analysis of variance test of methamphetamine and amphetamine concentration data observed in various tissues at two and four hours after the administration (IV) of benzphetamine

Parameter	Pair compared		Mean difference	F	p-Value
AP/MA[a]				18.7	<0.001
Tissue[a]				43.1	<0.001
2-H/4-H[a]				14.8	<0.001
Drug type	AP	MA	255		<0.001
Tissue[b]	Heart	Liver	-352.6		0.001
		Brain	-315.5		0.004
		Serum	92.2		1
		Kidney	-1012		<0.001
	Liver	Heart	352.6		0.001
		Brain	37		1
		Serum	444.8		<0.001
		Kidney	-659.4		<0.001
	Brain	Heart	315.5		0.004
		Liver	-37		1
		Serum	407.7		<0.001
		Kidney	-696.5		<0.001
	Serum	Heart	-92.2		1
		Liver	-444.7		<0.001
		Brain	-407.7		<0.001
		Kidney	-1104		<0.001
	Kidney	Heart	1012		<0.001
		Liver	659.4		<0.001
		Brain	696.5		<0.001
		Serum	1104		<0.001
Time	2-H	4-H	227.3		<0.001

[a] The difference of metabolites concentration between different group were adjusted by multivariate analysis of variance analysis.

[b] The significance level of multiple comparisons was adjusted by Bonferroni adjustment. (α level divided by comparisons-times and the result less than 0.0125 was significant.)

Conclusions

Analysis of MA and AP in various rat tissues collected at two and four hours after the administration (IV) of BZ concluded: (a) AP is present at a concentration higher than MA, indicating *N*-demethylation of BZ as a competitive metabolic route; (b) the concentrations of AP and MA in these tissues are in the order of kidney > brain ~ liver > heart ≥ serum.

Acknowledgement

The authors are grateful to the financial support provided by the (Taiwanese) National Bureau of Controlled Drugs, Department of Health (DOH94-NNB-1003), the Institute of Forensic Medicine, Ministry of Justice, Republic of China under Grant No. IFM91-T05 and Ministry of the Interior Research Project (Grant No.100-0805-06-04-01).

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