Study on Analytical Methods of Multiple Nitrosamines in Sartan Pharmaceuticals

Shu-Han Chang, Ching-Chia Chang, Hui-Yu He, Li-Jing Wang, Shu-Yu Fan, You-Yu Lee, Jian-Lin Wu, Ya-Hui Hsu, **Mei-Chih Lin**, Su-Hsiang Tseng, Der-Yuan Wang

Division of Research and Analysis, Food and Drug Adminstration (TFDA)

Ministry of Health and Welfare, ROC (Taiwan)

1. Introduction

In June 2018, TFDA received the alarm from abroad about the incident for the contamination of valsartan active pharmaceutical ingredients (APIs). It was proved that a probable human carcinogen *N*-Nitrosodimethylamine (NDMA) was detected in valsartan API produced by Zhejiang Huahai.

NDMA is a member of the class of *N*-nitrosamines, which is also classified in IARC Group 2A (The agent is probably carcinogenic to humans). Generally, nitrosamines are found in both natural conditions and in industrial manufacturing applications such as pesticides, rubbers, dyes, and pharmaceuticals. In industry, it is conjectured that they are formed as by-products in manufacturing processes using materials such as amines, nitrates, and nitrites under a range of pH conditions from different mechanisms, such as nitrosation by nitrite. A recent issue for unexpected detection of NDMA, NDEA and NMBA in sartan medicines has been reported, which a threat has raised for safety of mediation in many countries. To inspect the situation of contamination for sartan medicines in Taiwan, it is necessary to develop an analytical method in order to monitor the nitrosamine impurities. However, many studies have focused on the determination of nitrosamines in tobacco, food, water and cosmetics. Instead, an analytical method of nitrosamines detection in pharmaceuticals, such as sartans, still remains vacancy and is urgent to be developed. In this study, it was aimed to establish a feasible and sensitive LC-MS/MS and GC-MS/MS method for determination of 17 nitrosamines (Figure 1) in sartans.

2. Materials and Methods

2.1 Chemicals and reagents

N-Nitrosodibutylamine (NDBA), *N*-Nitrosodiethylamine (NDEA), *N*-Nitrosodimethylamine (NDMA), *N*-Nitrosodipropylamine (NDPA), *N*-Nitrosodimethylamine-d_6 (NDMA-d_6) and *N*-Nitrosodipropylamine-d_{14} (NDPA-d_{14}) were purchased from AccuStandard (CT, USA). *N*-Nitrosoethylisopropylamine (NEIPA) was purchased from BOC Sciences (NY, USA). *N*-Nitrosodiisopropylamine (NDiPA) was purchased from Chem Service (PA, USA). *N*-Nitrosodiethanolamine (NDELA), *N*-Nitrosomorpholine (NMOR) and *N*-Nitrosopyrrolidine (NPYR) were purchased from Sigma-Aldrich (MO, USA). *N*-Nitrosodiphenylamine (NDPhA) and *N*-Nitrosopiperidine (NPIP) were purchased from Supelco (PA, USA). *N*-Nitrosodicyclohexylamine (NDCHA), *N*-Nitrosodiisobutylamine (NDiBA), *N*-Nitrosodiisononylamine (NDiNA), *N*-Nitrosodiisopropanolamine (NDiPLA), *N*-Nitrosomethylethylamine (NMEA), *N*-Nitroso-*N*-methyl-4-aminobutyric acid (NMBA), *N*-Nitrosodiethylamine-d_4 (NDEA-d_4), *N*-Nitrosodiphenylamine-d_{10} (NDPhA-d_{10}), *N*-Nitroso-*N*-methyl-4-aminobutyric acid-d_3 (NMBA-d_3) were purchased from TRC (ON, Canada).

Methanol and formic acid of LC-MS grade were purchased from Sigma-Aldrich (MO, USA). Acetonitrile of LC-MS grade was purchased from J.T Baker (NJ, USA). Water of LC-MS grade was purchased from Scharlau (Barcelona, Spain).

2.2 Equipment

2.2.1 LC-MS/MS

- Waters Acquity UPLC system
- AB SCIEX QTRAP 6500

➢ **LC column**

- Waters XSelect HSS T3 column (15 cm × 3 mm i.d., 3.5 μm)

➢ **LC-MS/MS conditions**

- Sample injection: 10 μL
- Column temperature: 40℃
- Flow rate: 0.6 mL/min
- Mobile phase: Gradient analysis
- A: 0.1% formic acid in water
- B: 0.1% formic acid in acetonitrile/methanol (2:8)
- Detection time: 0.0 - 8.5 min
- Ion source: APCI+
- Nebulizer current: 5 μA
- Curtain gas: 25 psi
- Ion source gas: 50 psi
- Temperature: 400℃
- Detection mode: sMRM (Table 1)

2.2.2 GC-MS/MS

- Agilent Technologies System (GC 7890B, MS 7000C Triple Quad)

➢ **GC column**

- Agilent DB-WAX Ultra Inert column (30 m × 0.25 mm i.d., 0.25 μm)

➢ **GC-MS/MS conditions**

- Sample injection: 2 μL
- Injection mode: pulsed splitless mode
- Injection temperature: 250℃
- Interface temperature: 250℃
- Carrier gas: He
- Flow rate: 1.0 mL/min
- Oven temperature: 80℃ (3 min) →250℃ at 20℃/min (3 min)
- Electronic impact (EI): 70 eV
- EI source temperature: 230℃
- Q1 and Q2 temperature: 150℃
- Detection mode: MRM (Table 1)

2.3 Analytical procedure

2.3.1 Sample preparation for GC-MS/MS analysis

Sample 100 mg
↓Add 500 μL of IS (20 ng/mL)
↓Add methanol to 5 mL
↓Sonicate (30 min)
↓Filter (0.22 μm PVDF)

2.3.2 Sample preparation for LC-MS/MS analysis

Sample 250 mg
↓Add 250 μL of IS (400 ng/mL)
↓Add methanol 250 μL
↓Votex and Sonicate (5 min)
↓Add water 4.5 mL
↓Votex and Sonicate (5 min)
↓Centrifuge 3000 × g (5 min)
↓Filter (0.22 μm PVDF)

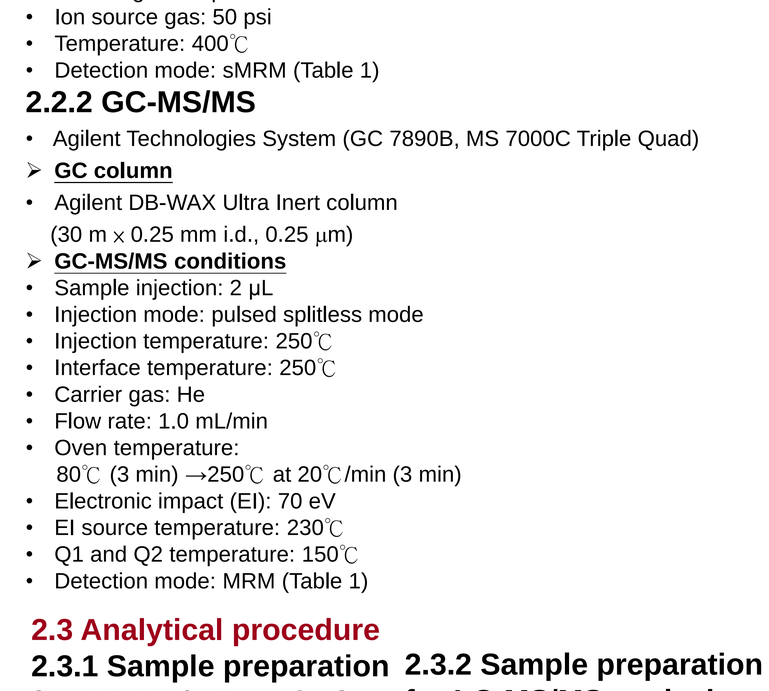


Figure 1. Chemical structures of the 17 nitrosamines and 6 internal standards.

Table 1. MRM transitions of compounds included in the methods.

No.	Compound	M.W.	GC-MS/MS		LC/MS-MS	
			Quantifier	Qualifier	Quantifier	Qualifier
1	NDMA	74	74>42	74>44	75>58	75>43
2	NDEA	102	102>85	102>56	103>75	103>47
3	NMBA	146	-	-	147>117	147>87
4	NDiPA	130	130>88	116>44	131>89	131>43
5	NEIPA	116	116>99	88>71	117>75	117>47
6	NDELA	134	-	-	135>74	135>104
7	NDiPLA	162	-	-	163>88	163>70
8	NDPA	130	130>113	130>43	131>89	131>43
9	NMEA	88	88>71	88>42	89>61	89>29
10	NMOR	116	116>86	116>56	117>87	117>86
11	NPIP	114	114>84	114>97	115>69	115>41
12	NPYR	100	100>55	100>70	101>55	101>41
13	NDBA	158	116>99	158>99	159>57	159>103
14	NDiBA	158	115>84	103>57	159>57	159>103
15	NDiNA	298	169>99	218>225	299>57	299>71
16	NDCHA	210	210>128	210>111	211>129	211>83
17	NDPhA	198	169>168	169>167	-	-
18	NDMA-d_6	80	80>46	-	81>46	-
19	NDEA-d_4	106	106>88	-	107>77	-
20	NMBA-d_3	149		-	150>120	-
21	NDPA-d_{14}	144	110>78	-	145>50	-
22	NDPhA-d_{10}	208	179>177	-	-	-
23	NDELA-d_8	142	-	-	143 > 111	-

3. Results and Discussion

3.1 LC-MS/MS

For the LC-MS/MS system, 16 nitrosamines standard can be analysed excluding NDPhA, which is decomposes during ionization. However, 4 nitrosamines (NDBA, NDiBA, NDiNA, NDCHA) coelute with the sartan ingredient and their signals are suppressed. The remaining 12 nitrosamines could be detected simultaneously in spiked sartan samples (candesartan, irbesartan, losartan, olmesartan and valsartan). In this method, 12 nitrosamines and 5 internal standards elute within 8.5 minutes (Figure 2), and the total run time is 15 minutes to flush out the high concentration of sartan matrix. The linearity from 2.5 to 50 ng/mL (IS 20 ng/mL) was evaluated by the R value, and R was above 0.9977 for all compounds. The recovery of intra-day and inter-day was 87.5-113.9 % for spiked samples in 5 different sartan APIs, while the range of precision was 2.9 - 12.1 % (Table 2). However, only the recovery of NDiPA, NDPA and NEIPA in irbesartan API was not within the range of 100.0 ± 20.0 %. The limit of quantitation (LOQ) for 12 nitrosamines were 0.05 μg/g.

3.2 GC-MS/MS

For the GC-MS/MS system, 16 nitrosamines standard can be analysed excluding NMBA. However, NDELA and NDiPLA signals are too weak and unable to achieve good sensitivity, so only 14 nitrosamines are suitable for GC-MS/MS analysis. In this method, 14 nitrosamines and 4 internal standards could be detected simultaneously within 14.5 minutes (Figure 3). The linearity from 1 to 50 ng/mL (IS 20 ng/mL) was evaluated by the R value, and R was above 0.9995 for all compounds. The recovery of intra-day and inter-day was 97.0-104.8 % for spiked samples in 5 sartan APIs, while the range of precision was 0.5 - 12.2 % (Table 3). Only the recovery of NPYR in low concentration of olmesartan and valsartan API exceeds 120%. The limit of quantitation (LOQ) for 14 nitrosamines were 0.05 μg/g.

The two methods complement each other and the compounds that have less acceptable recoveries in either method can be compensated and measured by the other methods.

3.3 Analysis of sartan samples

The methods are being applied to analyze 181 drug substances and 419 drug products, including valsartan, losartan, irbesartan, candesartan, olmesartan, telmisartan, azilsartan and pioglitazone pharmaceuticals (Figure 4). The test results showed that NDMA was detected in valsartan; NDEA was detected in irbesartan, losartan and valsartan. NMBA was detected in losartan and valsartan. NMOR, NPIP and NDPhA were also detected in valsartan. When these impurities were found in the APIs, the corresponding drug products were also found to contain these impurities.

4. Conclusions

Two fast and sensitive methods were developed using LC-MS/MS and GC-MS/MS techniques to detect 17 nitrosamines at low concentration in sartan drugs. The optimized methods are completed within 15 minutes with good reproducibility and recovery. More work will be done to further improve the sensitivity and recovery of the two methods in the future.

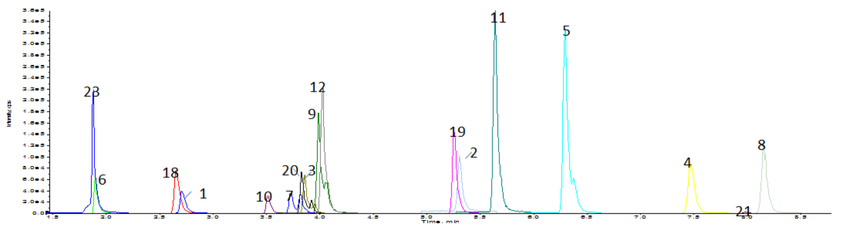


Figure 2. The MRM chromatograms of 12 nitrosamines and 5 internal standards analyzed by LC/MS/MS. (The peak number corresponding to peak name is shown in Table 2)

Table 2. LC/MS/MS – Accuracy and precision expressed as the recovery (%) and relative standard deviation (RSD in %) of the mean of analysis performed on at least three different days.

Compounds	R	Spiked concentration	Intraday (n=3, , 5 matrices)		Interday (n=9, , 5 matrices)	
		(ng/mL)	Accuracy	Precision	Accuracy	Precision
NDMA	0.9988	2.5	101.6	6.4	101.5	7.7
		25	106.0	4.7	104.9	3.8
		50	102.6	3.1	103.3	4.2
NDEA	0.9994	2.5	107.5	5.0	110.3	5.3
		25	106.6	4.5	106.3	5.0
		50	104.2	6.5	104.1	6.2
NMBA	0.9977	2.5	107.9	6.2	108.1	6.0
		25	100.4	7.8	99.1	7.8
		50	99.5	7.7	98.3	6.5
NDiPA*	0.9994	2.5	95.9	8.8	97.1	8.8
		25	104.3	5.9	106.3	7.0
		50	105.7	7.1	106.4	7.6
NEIPA*	0.9992	2.5	98.7	8.5	100.2	10.1
		25	97.7	11.1	100.3	12.1
		50	96.1	7.0	97.0	7.7
NDELA	0.9989	2.5	99.5	6.8	100.5	7.1
		25	105.4	3.8	106.1	4.0
		50	104.7	5.1	106.4	5.2
NDiPLA	0.9995	2.5	106.1	2.9	108.4	5.7
		25	98.8	6.6	102.8	7.7
		50	100.7	5.0	100.9	6.6
NDPA*	0.9996	2.5	89.2	6.4	87.5	6.8
		25	99.2	8.7	100.7	8.6
		50	100.9	5.0	100.8	7.1
NMEA	0.9994	2.5	95.7	6.4	94.4	6.7
		25	96.1	3.8	93.5	5.4
		50	97.5	9.0	93.1	8.5
NMOR	0.9993	2.5	107.9	5.9	110.1	6.0
		25	104.4	6.1	103.7	6.7
		50	108.9	5.8	103.7	7.6
NPIP	0.9983	2.5	111.0	5.8	113.9	4.5
		25	102.0	8.0	104.2	9.4
		50	98.7	7.2	101.6	7.9
NPYR	0.9992	2.5	95.6	5.1	92.1	6.5
		25	95.2	5.8	92.3	7.2
		50	96.7	4.9	91.9	6.4

* Only data from samples spiked into 4 different matrices are shown.

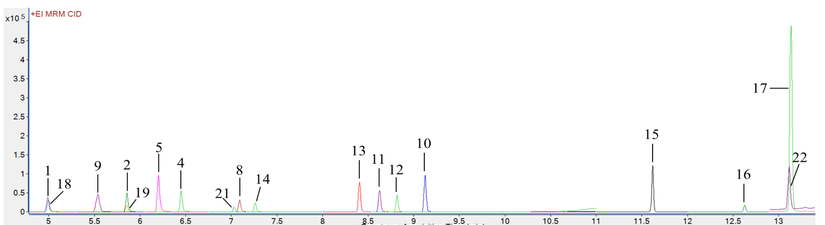
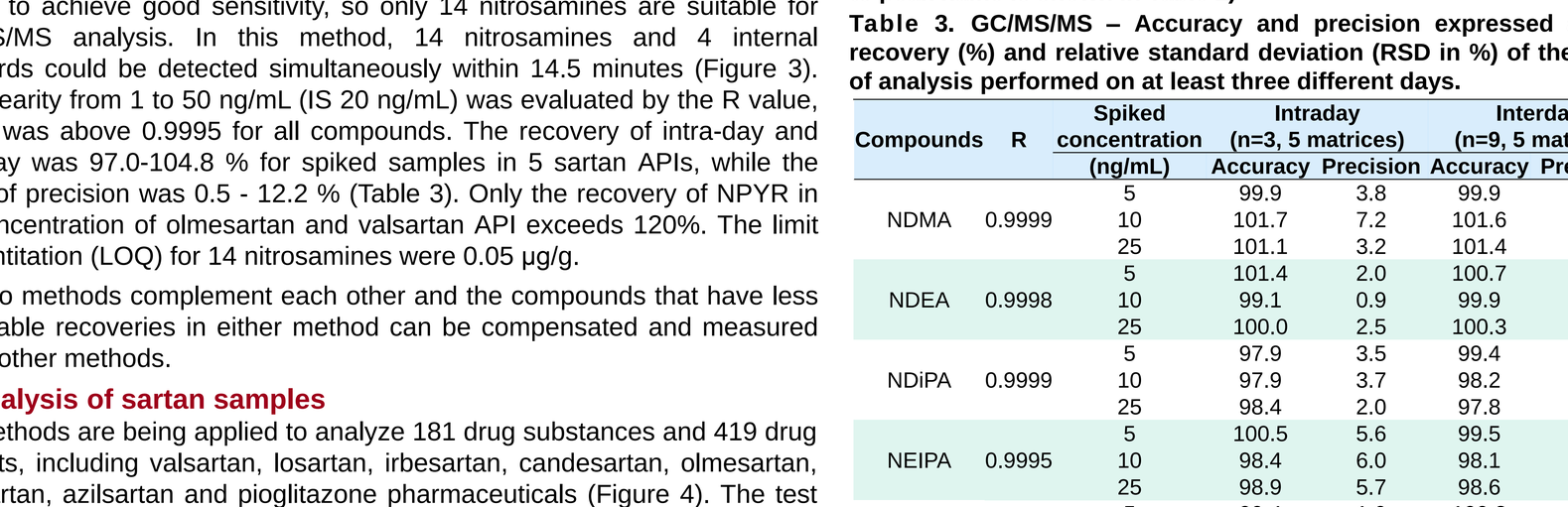


Figure 3. The MRM chromatograms of 14 nitrosamines and 4 internal standards analyzed by GC/MS/MS. (The peak number corresponding to peak name is shown in Table 2)

Table 3. GC/MS/MS – Accuracy and precision expressed as the recovery (%) and relative standard deviation (RSD in %) of the mean of analysis performed on at least three different days.

Compounds	R	Spiked concentration	Intraday (n=3, 5 matrices)		Interday (n=9, 5 matrices)	
		(ng/mL)	Accuracy	Precision	Accuracy	Precision
NDMA	0.9999	5	99.9	3.8	99.9	4.8
		10	101.7	7.2	101.6	4.9
		25	101.1	3.2	101.4	3.0
NDEA	0.9998	5	101.4	2.0	100.7	2.0
		10	99.1	0.9	99.9	1.5
		25	100.0	2.5	100.3	2.0
NDiPA	0.9999	5	97.9	3.5	99.4	3.8
		10	97.9	3.7	98.2	4.7
		25	98.4	2.0	97.8	3.3
NEIPA	0.9995	5	100.5	5.6	99.5	4.7
		10	98.4	6.0	98.1	5.9
		25	98.9	5.7	98.6	5.8
NDPA	0.9997	5	99.4	1.0	100.8	2.0
		10	100.3	0.8	101.5	1.9
		25	102.2	0.9	101.7	1.8
NMEA	0.9999	5	103.3	2.2	102.0	2.3
		10	99.8	3.0	99.9	2.7
		25	100.5	2.7	101.2	3.0
NMOR	0.9998	5	97.0	4.7	99.3	5.4
		10	97.5	3.2	98.6	4.8
		25	98.2	3.9	99.0	4.6
NPIP	0.9998	5	99.4	3.5	100.9	3.6
		10	99.1	3.9	100.1	3.6
		25	100.5	3.2	100.6	2.9
NPYR*	0.9998	5	103.6	3.0	102.1	3.7
		10	98.3	3.9	99.2	3.8
		25	100.1	5.4	99.7	4.3
NDBA	0.9998	5	102.3	4.0	104.1	4.4
		10	102.4	3.6	103.7	3.1
		25	103.0	4.4	103.0	4.0
NDiBA	0.9999	5	102.6	2.6	102.2	2.9
		10	101.7	3.4	102.4	3.1
		25	102.6	2.5	102.4	2.2
NDiNA	0.9998	5	100.6	6.6	101.1	10.8
		10	98.9	7.3	99.1	11.6
		25	99.7	9.7	100.2	12.2
NDCHA	0.9998	5	99.9	6.0	100.5	7.0
		10	98.5	4.1	98.8	6.7
		25	99.0	5.5	98.9	7.4
NDPhA	0.9998	5	103.5	5.6	104.8	5.9
		10	101.2	3.3	102.2	3.4
		25	100.8	0.5	101.8	2.2

* Only data from samples spiked into 3 different matrices are shown.



	Valsartan	Losartan	Irbesartan	Olmesartan	Candesartan	Telmisartan	Azilsartan	Pioglitazone
Total cases	192	266	63	26	10	19	19	5
Detected	56	36	5	0	0	0	0	0
NDMA detected	53	0	0	0	0	0	0	0
NDEA detected	11	4	5	0	0	0	0	0
NMBA detected	1	32	0	0	0	0	0	0
NMOR detected	2	0	0	0	0	0	0	0
NPIP detected	2	0	0	0	0	0	0	0
NDPhA detected	1*	-*	-*	-*	-*	-*	-*	-*

Figure 4. The testing results of *N*-nitrosamines determination in sartan active pharmaceutical ingredient and finished drug products in Taiwan.
*To be determined.