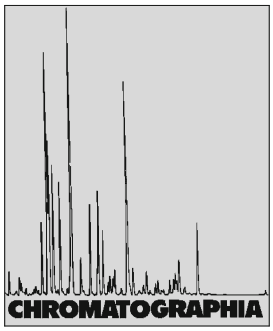
Simultaneous Determination of Levodopa, Benserazide and 3-*O*-Methyldopa in Human Serum by LC–MS–MS



2010, *72*, 627–633

Le Pan[1], Yanping Guo[2], Zhongdong Li[2,✉], Jun Chen[1], Tian Jiang[1], Yunqiu Yu[1,✉]

[1] School of Pharmacy, Fudan University, Shanghai 201203, People's Republic of China; E-Mail: yqyu@shmu.edu.cn
[2] Department of Pharmacy, Huashan Hospital, Fudan University, Shanghai 20040, People's Republic of China; E-Mail: Zdli@sh163.net

Received: 14 April 2010 / Revised: 13 June 2010 / Accepted: 29 June 2010
Online publication: 22 August 2010

Abstract

For the first time a sensitive, specific and rapid LC–MS–MS assay is presented for the simultaneous determination of levodopa (L-DP), 3-*O*-methyldopa (3-OMD) and benserazide (BSZ) in human serum. The three compounds were extracted from human serum by protein precipitation followed by dilution of the supernatant with aqueous formic acid. In serum, linearity was observed between 50 and 1,000 ng mL^{-1} of L-DP, 3-OMD and BSZ, respectively. Intra-day and inter-day RSD values were below 10.56 and 6.22% at concentrations of 120, 360 and 720 ng mL^{-1}. The presented method showed excellent specificity and sensitivity compared with other methods reported. It was applied to a pharmacokinetic study and demonstrated its applicability to pre-clinical and clinical pharmacological research.

Keywords

Column liquid chromatography-tandem mass spectrometry
Levodopa
Benserazide
3-*O*-methyldopa

Introduction

Levodopa, (-)-3-(3,4-dihydroxyphenyl)-L-alanine (L-DP), is the immediate precursor for the neurotransmitter dopamine. Unlike dopamine, L-DP easily enters the central nervous system, hence being used in the treatment of Parkinson's disease, which is associated with dopamine depletion in the brain [1, 2]. In vivo, L-DP is converted by catechol-*O*-methyltransferase (COMT) to 3-*O*-methyldopa (3-OMD) [3] and the concentration of 3-OMD has great influence on the metabolism and transport of L-DP.

In a therapeutic regimen high doses of L-DP are required due to rapid decarboxylation which means that very little unchanged drug is available to cross the blood–brain barrier for central conversion into dopamine. Consequently, L-DP is usually associated with a peripheral decarboxylase inhibitor, and benserazide, (*R*/*S*)-2-amino-3-hydroxy-2′-(2,3,4-trihydroxybenzyl)propanohydrazide (BSZ) is the first choice [2].

Hence, for quality control of pharmaceutical formulations, it is important to develop a method for the selective and simultaneous determination of L-DP, 3-OMD and BSZ. Few methods are available for the simultaneous determination of these three drugs. Currently existing methods include quantitative application to L-DP and 3-OMD analysis using electrochemical detection [4–6] or simultaneous determination of L-DP and BSZ in vivo using differential spectrophotometry [7], derivative spectrophotometry [8–10] and kinetic-spectrophotometric methods [11, 12]. Within the context of a clinical pharmacokinetics study, however, higher specificity and sensitivity are required.

Atmospheric pressure ionization mass spectrometry (API-MS) techniques, such as electrospray (ESI), had a large impact on bio-analytical chemistry by providing a highly selective, sensitive, and robust technique for the detection and quantitation of a wide variety of compounds following appropriate analytical separation [13, 14]. The most

Original
DOI: 10.1365/s10337-010-1683-x
0009-5893/10/10

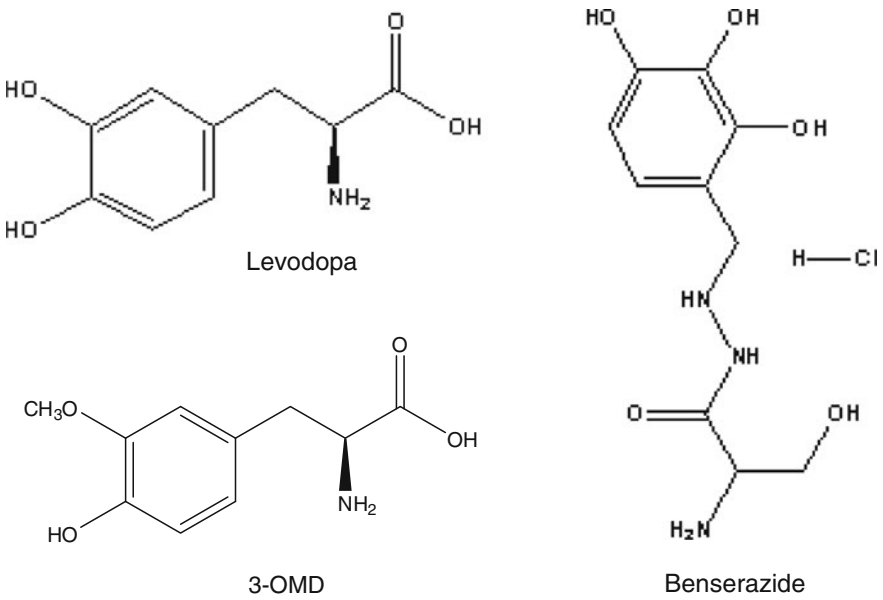


Fig. 1. Chemical structures of levodopa, benserazide and 3-OMD

Table 1. MS–MS conditions

Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	DP (V)	FP (V)	CE (V)	CXP (V)
212.3 (3-OMD)	149.0	44	150	22	10
258.2 (BSZ)	85.2	26	110	36	9
198.3 (L-DP)	152.0	44	120	20	9
227.3 (Carbidopa)	180.9	45	110	18	12

DP declustering potential; FP focusing potential; EP entrance potential; CE collision energy; CXP collision cell exit potential

dramatic impact of this approach has been the drastic reduction in method development time for ionizable analytes due to substantial improvement in selectivity offered by tandem mass spectrometry [15].

This paper reports on a new bioanalytical methodology for the simultaneous quantitation of L-DP, 3-OMD and BSZ in human serum using liquid chromatography-electrospray tandem mass spectrometry (ESI-LC–MS–MS). This method was developed in order to allow for a convenient, specific, sensitive and reproducible approach to therapeutic drug monitoring of L-DP, 3-OMD and BSZ.

Experimental

Materials

L-DP (99.5%), 3-OMD (99.3%), BSZ hydrochloride (98.3%), and carbidopa (99%) were purchased from Sigma (Steinheim, Germany). Chemical structures of test compounds are given in Fig. 1. Solvents and reagents, such as methanol (HPLC, 99.9%) and formic acid (HPLC, 99.9%), were purchased from Dikma (Richmond Hill, ON, Canada).

Liquid Chromatography Mass Spectrometry

Assays were performed on an Agilent 1100 Series HPLC (Palo Alto, USA) with a PE Sciex API 3000 triple quadrupole mass spectrometer. A YMC PACK MB-ODS (150 mm × 2.1 mm, 3 µm) reversed-phase column was used. Methanol:0.5% formic acid (30:70, *v*/*v*) was used as the mobile phase at a flow rate of 0.20 mL min^{-1}. The mass spectrometer was equipped with a turbo-ionspray source. The turbo-ionspray temperature was set at 500 °C. Quantitation was performed using multiple reaction monitoring (MRM). Ion transitions and instrumental parameters are shown in Table 1.

Standard Solution Preparation

Stock solutions of L-DP, 3-OMD, BSZ and internal standard (carbidopa) were prepared by accurately weighing 10 mg of compound followed by dissolution with 0.1% aqueous formic acid and dilution to volume using a 10 mL volumetric flask. Concentrations were adjusted for percent purity and percent free base by filtering through a 0.22-µm filter (Turner Science Institute, Tianjin, China).

The working stock solution was made by mixing the three stock solutions of L-DP, 3-OMD and BSZ to give a concentration of 250 µg mL^{-1}. A series of working standard solutions were prepared by diluting the working stock solution with different amount of 0.1% aqueous formic acid to give concentrations of 500, 1,000, 2,000, 3,000, 5,000, 8,000 and 10,000 ng mL^{-1}, respectively.

Serum-based calibration solutions were prepared by using the dilution standard solution procedure to yield final concentrations of 50, 100, 200, 300, 500, 800 and 1,000 ng mL^{-1}, respectively.

Three other standard solutions of 1,200, 3,600 and 7,200 ng mL^{-1} were also prepared, independently, as quality control samples, and were diluted to yield low (120 ng mL^{-1}), medium (360 ng mL^{-1}), and high (720 ng mL^{-1}) control concentrations in human serum.

The internal standard (IS) carbidopa stock solution was also diluted to 1 µg mL^{-1} as working IS solution for next step.

Sample Preparation

The pre-processing of the spiked serum samples or clinical samples included three major steps. First, to 100 µL of serum were added 30 µL working IS

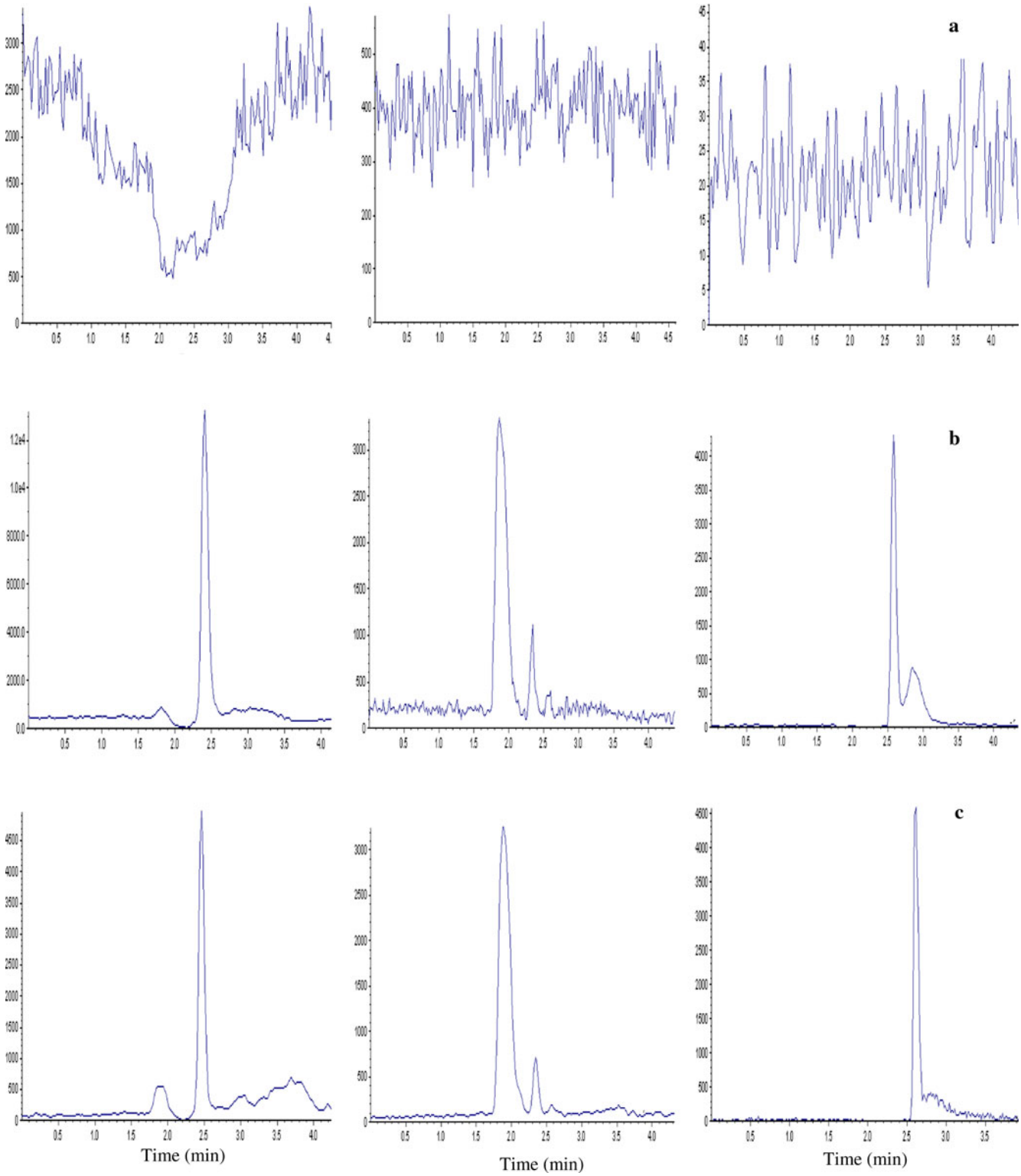


Fig. 2. **a** Blank serum sample; **b** spiked serum sample; **c** typical chromatograms obtained from volunteer serum. From left to right: L-DP (198.3 > 152.0), BSZ (258.2 > 85.2) and 3-OMD (212.3 > 149.0)

solution and mixed for 10 s. Second, 300 μL 2% (*v*/*v*) formic acid methanol solution was added to the serum and centrifuged at 14,000×*g* for 10 min at 4 °C. Finally, 100 μL supernatant was collected and diluted with 100 μL 0.1% aqueous formic acid (*v*/*v*) followed by LC–MS-MS analysis with a 5 μL aliquot.

Table 2. Accuracy and precision values for L-DP, BSZ and 3-OMD

Compound	Nominal Conc. (ng mL^{-1})	Intra-day ($n = 5$)		Inter-day ($n = 3$)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
L-DP	50	104.2	13.42	101.5	6.45
BSZ		105.6	12.12	99.8	7.68
3-OMD		102.3	8.93	102.3	5.10
L-DP	120	101.0	10.56	99.5	1.90
BSZ		98.2	8.56	100.2	2.30
3-OMD		97.2	5.58	101.7	3.61
L-DP	360	100.8	7.34	99.7	1.17
BSZ		99.7	6.24	99.8	1.90
3-OMD		100.0	8.19	100.3	0.74
L-DP	720	98.7	7.56	100.1	4.12
BSZ		98.5	6.02	100.7	2.40
3-OMD		99.1	7.70	100.8	6.22

Table 3. Recoverie values for L-DP, BSZ and 3-OMD

Compound	Nominal Conc.	Recovery (%)	RSD (%)
L-DP	120 ng mL^{-1}	57.3	7.81
BSZ		47.8	8.82
3-OMD		60.1	4.10
L-DP	360 ng mL^{-1}	54.2	3.24
BSZ		49.3	6.72
3-OMD		61.2	4.21
L-DP	720 ng mL^{-1}	55.0	2.39
BSZ		48.6	4.55
3-OMD		60.4	3.18
Carbidopa (IS)	1 μg mL^{-1}	61.2	4.34

Table 4. Pharmacokinetic parameters of L-DP and 3-OMD

Parameters	L-DP		3-OMD	
	Test	Reference	Test	Reference
C_{max} (μg mL$^{-1)}$	0.56	0.6	0.62	0.65
t_{max} (h)	3.0	3.0	7.0	6.5
$t_{1/2}$ (h)	1.8	1.6	N/A	N/A
$AUC_{0\text{-}t}$ (μg h mL^{-1})	1.79	1.83	9.3	9.62
F (%)	95.0		96.3	

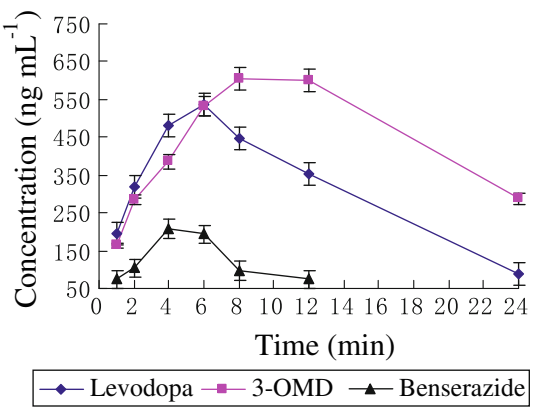


Fig. 3. Mean plasma concentration-time profiles of L-DP, BSZ and 3-OMD after oral administration of a single dose

Linearity and LLOQ

The linearity was determined by injecting seven serum-based calibration samples (50, 100, 200, 300, 500, 800 and 1,000 ng mL^{-1} in blank human serum) according to the sample preparation procedure described above.

LLOQ was the lowest standard on the calibration curves which was 50 ng mL^{-1}.

Accuracy and Precision

The intra-day accuracy and precision tests were carried out at LLOQ level (50 ng mL^{-1}). Three typical concentrations in human serum (120, 360, and 720 ng mL^{-1}) were analysed using five replicate samples. The inter-day tests repeated carried out the same procedure on three successive days.

Recovery efficiency was evaluated using three separate concentrations in serum (120, 360, and 720 ng mL^{-1}). The peak area of L-DP, 3-OMD and BSZ obtained from spiked serum after pre-processing were compared to the peak area obtained from the nominal concentration of the three compounds. The recovery of IS was also tested at concentration of 1 μg mL^{-1}.

Matrix Effect

Matrix effect was evaluated using the three standard solutions of 1,200, 3,600 and 7,200 ng mL^{-1}. The three solutions were diluted to 120, 360, and 720 ng mL^{-1}, respectively, with 0.1% aqueous formic acid (v/v) and the supernatant was collected from blank serum sample after protein precipitation. Then the two kinds of solutions were injected for LC–MS–MS analysis. Finally, the peak areas of L-DP, 3-OMD and BSZ obtained from 0.1% aqueous formic acid solutions were compared to the peak areas obtained from the three compounds derived from the supernatant solution, respectively.

Results and Discussion

Specificity

LC–MS chromatograms obtained from L-DP, 3-OMD and BSZ for a blank serum sample are shown in Fig. 2a, and a spiked serum sample (concentration at 100 ng mL^{-1} for L-DP, 3-OMD and BSZ, respectively) are shown in Fig. 2b. Typical chromatograms obtained from volunteer serum are displayed in Fig. 2c. The retention times were 2.5 min for L-DP, 2.0 min for BSZ and 2.6 min for 3-OMD.

The chromatograms showed that L-DP, 3-OMD, BSZ and IS were not detected in drug-free serum. The chromatograms did not show any additional peaks due to endogenous substances or other metabolites that might have interfered with drug detection at the same retention times.

Linearity and LLOQ

Calibration curves were constructed by plotting the peak area ratios of L-DP, 3-OMD, BSZ and IS versus concentration (ng mL^{-1}). The following linear equations were determined: $y = 0.0011x + 0.037$ ($r^2 = 0.997$) for L-DP; $y = 0.00075x + 0.014$ ($r^2 = 0.999$) for 3-OMD and $y = 0.00118x + 0.003$ ($r^2 = 0.996$) for BSZ, respectively. The regression equation was generated by employing the least square method. The LLOQ of the three compounds (serum sample solution at 50 ng mL^{-1}) were defined as the lowest concentration of the analytes that could be determined with accuracy values between 80 and 120% and a precision below 20% ($n = 5$).

Accuracy Precision and Recovery

Table 2 shows that the assay met the requirements set for method validation and Table 3 summarizes the recovery values obtained for L-DP, 3-OMD, BSZ and IS, respectively.

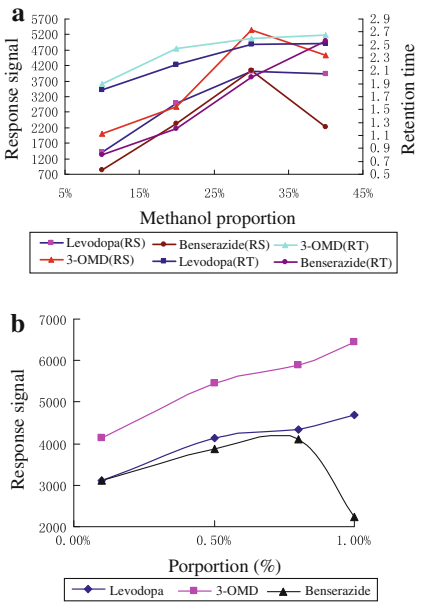


Fig. 4. Optimization of LC conditions. **a** Effect of methanol on retention time (RT) and signal response (RS); **b** effect of formic acid proportion on signal response

Matrix Effect

The matrix effect of the method was 75.69% for L-DP, 85.95% for 3-OMD and 69.70% for BSZ, respectively.

Pharmacokinetics Study

The validated assay of L-DP, 3-OMD and BSZ was used to support a pharmacokinetic study. The PK study on healthy male was performed according to the guidelines of the FDA and SFDA and was agreed by the ethics committee of Huashan hospital at Fudan University, Shanghai, China. A total of ten healthy young subjects were enrolled and each of them received an orally administered dose of a controlled-release capsule simultaneously (L-DP 100 mg/BSZ 25 mg). Blood samples were obtained before dosing and at 0, 2, 4, 6, 8, 10, 12, 24 h after administration.

Figure 3 shows the mean plasma concentration–time profiles of L-DP, 3-OMD and BSZ after single-dose administration. The main pharmacokinetic parameters (C_{max}, t_{max}, $AUC_{0\text{-}t\ max}$, $t_{1/2}$) of L-DP and

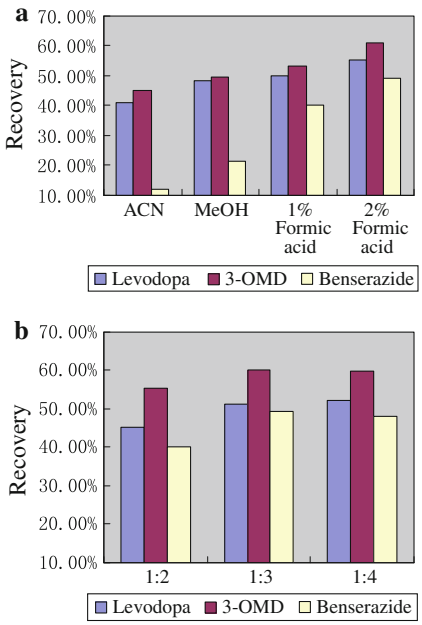


Fig. 5. Optimization of the sample pre-processing procedure. **a** Influence of different precipitation solvents on recovery; **b** influence of volume ratio of serum and 2% formic acid (v/v) in methanol

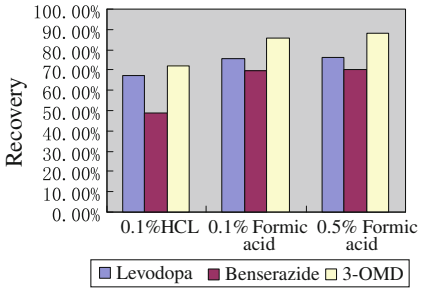


Fig. 6. Recovery values obtained from different acidified dilution solvents

3-OMD are shown in Table 4 and the relative bioavailability of the test tablets was 95.0% for L-DP and 96.3% for 3-OMD.

The results indicated that there were no significant differences found when compared with the reported literatures [3, 16] which demonstrated the applicability of the method to support preclinical pharmacokinetic studies.

Optimization of LC Conditions

LC conditions were optimized with respect to the methanol proportion and

Table 5. Comparison of qualitative and quantitative parameters between the presented study using LC–MS–MS and methods reported for the determination of LDP, BSZ and 3-OMD in vivo

	Simultaneous determination	Specificity (resolution)	Precision (%)	LLOQ (ng mL^{-1})	Analysis time
Electrochemical detection [4–6]	No	Poor (<1)	10	500	7 min
Differential spectrophotometry [7]	No	Fair (≈1)	15	1,000–1,500	30 min
Derivative spectrophotometry [8–10]	No	Fair (≈1)	12	1,000	2 h
Kinetic-spectrophotometric [11, 12]	No	/	10	500	6–8 h
LC–MS/MS method	Yes	Good (≈1)	<10	50	<5 min

formic acid proportion used in the aqueous phase. The effect of methanol on the retention time and response signal of the analytes was investigated by varying its content from 0% to 40% (*v*/*v*) in the mobile phase. As the proportion of methanol increased, the peak areas of the three compounds increased greatly. On the other hand, this resulted in high analyte retention and long analysis times (Fig. 4a).

Based on the high polarity of the three compounds the high proportion of aqueous solvent used increased retention times but lowered nebulisation efficiency. Conversely, increased methanol contents led to improved nebulization efficiency (Fig. 4a) which led to the decision to use a composition with 30% (*v*/*v*) methanol. The formic acid proportion in aqueous phase affected the signal response of L-DP, 3-OMD and BSZ remarkably. The three compounds were most stable under low pH conditions, so formic acid was added to the aqueous phase to prevent decomposition in the ESI source. Formic acid could help in the ionization of molecules, especially polar molecules [17–19]. A small amount of formic acid in the mobile phase would stabilize polar molecules in the ESI source, which then would convert to more $[M + H]^+$ species to increase signal response. The formic acid proportion studied ranged from 0.1 to 1% (*v*/*v*) Fig. 4b shows that the peak area of L-DP and 3-OMD increased as the amount of proportion increased from 0.1 to 1%. However, a sharp drop of BSZ intensity was observed when more formic acid was added. This was because of the three hydroxy groups and two amino groups present in the chemical structure of BSZ (Fig. 1) [2]. Finally, a 0.5% proportion was chosen for this study.

Optimization of Sample Pre-processing Procedure

The protein precipitation method (PP) is by far the easiest and fastest way of sample pre-treatment, especially in LC–MS–MS analysis [20], so methanol, acetonitrile, 1% (*v*/*v*) formic acid and 2% (*v*/*v*) formic acid in methanol were investigated as potential precipitation solvents (volume ratio serum and PP solvent was 1:3). The highest recovery and best reproducibility were obtained when using 2% (*v*/*v*) formic acid in methanol as summarized in Fig. 5a. which was then used for the study. The volume ratio of serum and 2% (*v*/*v*) formic acid in methanol was investigated and ranged from 1:2 to 1:4 (*v*/*v*). Figure 5b shows that highest recoveries were achieved when the volume ratio was 1:3.

Optimization of the Dilution Solvents

Once the protein precipitation method was chosen as the pre-processing procedure, it was observed that after approximately 300 injections of the supernatant, the HPLC column performance decreased dramatically. Analyte/internal standard ratios were not constant and MS signals dropped sharply. That might be for the accumulation of matrix effects in the whole system and supernatant, leading to decreased MS signal response. This situation was significantly improved when the supernatant was diluted with acidic components. For this purpose, 0.1% (*v*/*v*) HCl, 0.1% (*v*/*v*) formic acid and 0.5% (*v*/*v*) formic acid were investigated as dilution solvents. As shown in Fig. 6, recovery and optimal signal intensity were obtained when using 0.1% formic acid, presumably due to reduced matrix effects [21] and stabilized ionization of L-DP, 3-OMD and BSZ in the ESI source.

Table 5 provides a comparison of qualitative and quantitative parameters found in the present study and in previously reported applications. It was found that the presented LC–MS–MS showed improved capability, particularly important for pharmacokinetic studies.

Conclusions

A LC–MS–MS method was established for the simultaneous determination of L-DP, 3-OMD and BSZ. L-DP, 3-OMD and BSZ were extracted from human serum using protein precipitation. The method showed excellent specificity and sensitivity when compared with commonly used analytical methods and was validated for precision, accuracy, sensitivity and linearity. The method was easy to perform and was successfully employed in a pharmacokinetic study.

References

1. Muzzi C, Bertocci E, Terzuoli L, Porcelli B, Ciari I, Pagani R, Guerranti R (2008) Biomed Pharmacother 62:253–258
2. Marques KL, Santos JL, Lopes JA, Lima JL (2008) Anal Sci 24:985–991
3. Nunes T, Machado R, Rocha JF, Fernandes-Lopes C, Costa R, Torrão L, Loureiro AI, Falcão A, Vaz-da-Silva M, Wright L, Almeida L, Soares-da-Silva P (2009) Clin Ther 31:2258–2271
4. Blandini F, Martignoni E, Pacchetti C, Desideri S, Rivellini D, Nappi G (1997) J Chromatogr B Anal Technol Biomed Life Sci 700:278–282
5. Michotte Y, Moors M, Deleu D, Herregodts P, Ebinger G (1987) J Pharm Biomed Anal 5:659–664

6. Igarashi K, Hotta K, Kasuya F, Abe K, Sakoda S (2003) J Chromatogr B Anal Technol Biomed Life Sci 792:55–61
7. Davidson AG (1985) J Pharm Biomed Anal 3:235–240
8. Hassib ST (1990) Anal Lett 23:2195–2214
9. Uslua B, Özkana SA (2002) Anal Lett 35:303–314
10. Karpińska J, Smyk J, Wołyniec E (2005) Spectrochim Acta A 62:213–220
11. Pistonesi M, Centurión ME, Fernández Band BS, Damiani PC, Olivieri AC (2004) J Pharm Biomed Anal 36:541–547
12. Coello J, Maspoch S, Villegas N (2000) Talanta 53:627–637
13. Wu G, Furlanut M (1997) Pharmacol Res 35:553–556
14. Andreoli R, Manini P, Bergamaschi E, Mutti A, Franchini I, Niessen WM (1999) J Chromatogr A 847:9–17
15. Lee MS, Kerns EH (1999) Mass Spectrom Rev 18:187–279
16. Crevoisier C, Monrealc A, Metzgerc NilsenT (2003) Eur Neurol 49:39–44
17. Zhang D, Wang Y, Han J, Yu W, Deng L, Fawcett JP, Liu Z, Gu J (2009) J Chromatogr B Anal Technol Biomed Life Sci 877:581–585
18. Juan-García A, Font G, Picó Y (2007) Electrophoresis 28:4180–4191
19. Yüzüak N, Özden T, Erenl S, Toptan S (2007) Chromatographia 66:115–118
20. Damen CW, Rosing H, Tibben MM, van Maanen MJ, Lagas JS, Schinkel AH, Schellens JH, Beijnen JH (2008) J Chromatogr B Anal Technol Biomed Life Sci 868:102–109
21. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Anal Chem 75:3019–3030