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Analysis of benzodiazepines and their metabolites using DBS cards and LC–MS/MS

Heesang Lee [a], Yujin Park [a], Jiyeong Jo [a], Sangwhan In [a], Yonghoon Park [a], Eunmi Kim [a], Jaesung Pyo [b], Sanggil Choe [a,*]

[a] Busan Institute, National Forensic Service, 50 Geumhoro, Mulgeumeup, Yangsan, Republic of Korea
[b] College of Pharmacy, Kyungsung University, 309 Suyeongro, Namgu, Busan, Republic of Korea

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ABSTRACT

Dried Blood Spot (DBS) has been used a blood extraction method for inherited metabolic disorder screening since 1960s. With introduction of LC–MS/MS, not only DBS could be used to analysis drugs in small blood volume, but in various fields, such as toxicology, drug therapeutic monitoring, drug diagnostic screening, and illicit drugs. In toxicology field, many drugs (e.g. benzodiazepines, acetaminophen, small molecule drugs) have been tested with DBS. Compared with earlier blood extraction methods (SPE and LLE), DBS has lots of advantages; lower blood volume (less than 50 μL), shorter analysis time caused by a more concise analysis procedure and lower cost. We optimized the DBS procedure and LC–MS/MS conditions for 18 benzodiazepines, seven benzodiazepine metabolites, and one z-drug (zolpidem) analysis in blood. 30 μL of whole blood was spotted on FTA DMPK card C and dried for 2 h in a desiccator. A 6-mm disk was punched and vortexed for 1 min in a centrifuge tube with 300 μL methanol/acetonitrile mixture (1:1, v/v). After evaporation, redissolved in 100 μL mobile phase of LC–MS/MS and 5 μL was injected. In the analysis for 26 target compounds in blood, all of the method validation parameters – LLOD, LLOQ, accuracy (intra- and inter-assay), and precision (intra- and inter-assay) – were satisfied with method validation criteria, within 15%. The results of matrix effect, recovery, and process efficiency were good. We developed a fast and reliable sample preparation method using DBS for 26 benzodiazepines, benzodiazepine metabolites, and z-drug (zolpidem).

1. Introduction

Benzodiazepines, psychoactive drugs, have very effective sedation potency if they are used for short periods and have fewer adverse effects than other class of sedatives. Also, most benzodiazepines show their CNS-depressing effect within a few minutes. For all these reasons, benzodiazepines have been among the most prescribed drugs in the world over the past twenty years [1]. Benzodiazepines enhance the permeability of chloride ions through changing the activity of the $GABA_A$ receptor, a representative inhibitory receptor in the central nervous system, through binding to the benzodiazepines-binding site in the $GABA_A$ receptor. Benzodiazepines have been used for the treatment of anxiety, insomnia, agitation, seizures, muscle spasms, and alcohol withdrawal by inducing sedative, hypnotic, anxiolytic, anticonvulsant, and muscle relaxing effects by indirect inhibitory effects on nerve transmission. The z-drugs, the names of which start with the letter 'z' are a group of non-benzodiazepine drugs that show similar effects to benzodiazepines [2]. Zolpidem is a representative z-drug used for insomnia and some brain disorders [3].

Dried Blood Spot (DBS) is a blood extraction method that has been used for newborn screening (NBS) to confirm phenylketonuria (PKU) through analysis of phenylalanine in blood since 1963, due to Dr. Robert Guthrie [4]. This innovation had a huge effect over a long time and DBS has been used to diagnose genetic disorders for newborns in most countries worldwide. In its early days, there were limitations to its use in the forensic field because no equipment with sufficient sensitivity existed then. However, with the development of highly precise instruments like LC–MS/MS, DBS could be used in various areas, such as diagnostic screening [5–8], therapeutic drug monitoring [9–11], toxicokinetics [12], and pharmacokinetics [13,14].

DBS has many advantages when compared with other existing extraction methods (e.g., SPE and LLE) [15]. DBS is a less invasive sampling method; blood samples are collected through finger or heel pricks. Blood sample transportation and storage are simple

* Corresponding author. Tel.: +82 55 380 4111; fax: +82 55 380 4120.
E-mail address: csk91@korea.kr (S. Choe).

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and analyses are easy for many drugs: for example, benzodiazepines and cocaine [16,17]. Blood samples spotted on a DBS card can be stored for several months or years at room temperature if an appropriate humidity is maintained [13]. DBS can reduce the infection risk of HIV/AIDS and other infectious pathogens to a minimum by restricting the spread of transmissible diseases [18]. In performing DBS, very simple extraction procedure is a major point to help reduce the spread of infectious disease. Finally, the most important advantage is that it needs a very small blood sample volume and whole blood can be used for analysis with LC–MS/MS. While existing extraction methods need at least 0.5 mL blood volume, DBS is able to analyze drugs with less than 50 μL of blood [15]. As the requiring amount of blood sample volume for DBS was so low, DBS also presented a particular opportunity to reduce numbers of animal in experiments. DBS made it possible to collect blood samples from the same animal body and analyze toxicities of many drugs in blood simultaneously. DBS was a useful method for generating of toxicokinetics data in animal experimentation without individual variation [19].

In this paper, DBS card type, extraction solvent, extraction method, and extraction time for optimal procedures were selected for 18 benzodiazepines, seven benzodiazepine metabolites and one z-drug (zolpidem) and the analytical method for LC–MS/MS was fully validated.

2. Experimental

2.1. Chemical and reagents

HPLC-grade methanol and acetonitrile were purchased from AVANTOR Performance Materials Inc. (USA). Formic acid (98%, Fluka Analytical, Germany) and ammonium formate (Fluka

Table 1
LC–MS/MS conditions for target compounds and internal standard.

Target compounds	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	R.T (min)	DP	EP	CE	CXP
Alprazolam	309.1	*281.2* 205.1	3.31	96	4.5	33	4
Bromazepam	318.0	*182.1* 209.2	2.96	96	4.5	41	4
Chlordiazepoxide	300.1	*227.1* 282.2	2.67	86	4.5	31	4
Clobazam	301.1	*259.2* 224.2	3.51	96	3.5	25	6
Clonazepam	316.1	*270.2* 214.1	3.32	106	4.5	33	4
Diazepam	285.1	*154.1* 193.2	3.69	106	4.5	37	4
Estazolam	295.1	*267.2* 205.2	3.24	101	4.5	31	4
Flunitrazepam	314.1	*268.2* 239.1	3.46	106	4.5	33	4
Flurazepam	388.2	*315.1* 317.2	2.88	91	4.5	29	6
Lorazepam	321.1	*275.2* 303.1	3.28	106	4.5	27	4
Midazolam	326.1	*291.3* 249.2	2.85	111	4.5	35	4
Nitrazepam	282.1	*236.2* 180.2	3.24	91	4.5	33	4
Nordazepam	271.1	*140.2* 165.0	3.39	106	4.5	37	4
Oxazepam	287.1	*241.1* 269.1	3.22	96	4.5	31	4
Prazepam	325.1	*271.2* 140.1	4.08	101	4.5	29	4
Temazepam	301.1	*255.1* 283.0	3.46	96	4.5	27	4
Triazolam	343.1	*239.2* 308.1	3.35	106	3.5	53	4
Zolpidem	308.2	*235.1* 236.3	2.62	101	4.5	37	4
7-Aminoclonazepam	286.1	*121.0* 222.1	2.44	71	4.5	39	4
7-Aminoflunitrazepam	284.1	*135.2* 227.1	2.63	96	4.5	37	4
7-Aminonitrazepam	252.1	*121.1* 94.0	2.03	96	4.5	37	4
α-Hydroxyalprazolam	325.1	*297.2* 216.1	3.14	81	4.5	33	4
α-Hydroxymidazolam	342.1	*324.2* 203.1	2.84	106	4.5	27	6
α-Hydroxytriazolam	359.0	*331.1* 176.0	3.14	96	4.5	35	6
Desalkylflurazepam	289.1	*140.0* 226.2	3.41	101	4.5	41	4
Diazepam-d5	290.1	*227.0* 198.2	3.68	101	4.5	31	6

The quantification ions are in italic; R.T, retention time; DP, declustering potential; EP, entrance potential, CE, collision energy; CXP, collision exit potential.