Journal of Analytical Toxicology 2012;36:582–587
doi:10.1093/jat/bks064 Advance Access publication January 1, 2012

Article

Cross-Reactivity of Tapentadol Specimens with DRI Methadone Enzyme Immunoassay

Ayodele A. Collins, A. Paola Merritt and James A. Bourland*

Ameritox, Ltd., Midland, Texas

*Author to whom correspondence should be addressed. Email: jim.bourland@gmail.com

A substantial incidence of positive methadone screens for pain management urine specimens using a commercial enzyme immunoassay (EIA) was observed in the absence of a methadone prescription, with negative methadone confirmation by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS-MS). Tapentadol was the only common prescription among the investigated specimens. Tapentadol or one of its three major metabolites was tested at various concentrations (100–200,000 ng/mL) against the DRI EIAs for methadone and methadone metabolite, to evaluate cross-reactivity. Ninety-seven authentic tapentadol urine specimens that produced false-positive methadone EIA results (cutoff = 130 ng/mL) were analyzed for methadone and tapentadol in compound-specific UPLC–MS-MS confirmation tests. Tapentadol, tapentadol glucuronide, tapentadol sulfate and *N*-desmethyltapentadol exhibited cross-reactivity with the methadone EIA at 6,500 (2.2%), 25,000 (0.6%), 3,000 (4.4%) and 20,000 ng/mL (0.9%), respectively. No cross-reactivity was observed with the methadone metabolite 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine EIA. All authentic urine specimens were confirmed to be negative for methadone, but positive for tapentadol and all monitored metabolites. Individual concentrations indicated that separate or combined urinary concentrations of tapentadol and its conjugates may produce false-positive methadone screens through cross-reactivity with the methadone immunoassay. The potential for false-positive results for methadone EIA screening of urine specimens associated with tapentadol prescriptions should be considered when interpreting results.

Introduction

The DRI Methadone Assay (Microgenics Corp., Fremont, CA) is a homogeneous enzyme immunoassay (EIA) employed for preliminary analytical testing for the synthetic opioid methadone in urine. The detection of methadone by this immunoassay kit is based on competition between the drug in urine and enzyme-labeled drug in the EIA conjugate reagent for specific antibody binding sites in the EIA substrate reagent (1). Enzyme activity of the unbound enzyme label is directly related to the concentration of methadone in urine. Methadone (Mtd) is a narcotic analgesic commonly used in the management of severe chronic pain. It is also used in the detoxification and maintenance of heroin addicts because of its ability to suppress the craving for heroin without the euphoric effects of heroin. It is rapidly metabolized by *N*-demethylation to normethadone, which is readily dehydrated to form one of the major urinary excretion products, 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (2, 3). EDDP is not cross-reactive to the DRI Methadone Assay, but can be determined separately using the DRI Methadone Metabolite Assay (Microgenics Corp.). The DRI Methadone Metabolite Assay is not cross-reactive to methadone. The application of the metabolite screen helps to discriminate false-positive results from patients who add the drug exogenously to their urine, or false-negative results in which only the methadone metabolite may be present.

Immunoassays are typically employed as a presumptive screen preceding a confirmatory analysis in a two-tiered drug testing protocol. A distinct disadvantage of EIAs is that they lack specificity, which is attributable to the cross-reactivity of other non-targeted analytes to the specific drug antibody. The EIAs are susceptible to interferences from substances other than the targeted compound, yielding false-positive results (1, 4). Consequently, it is recommended that positive EIA results be confirmed by a more specific nonimmunological method, such as gas chromatography mass spectrometry (GC–MS) or liquid chromatography tandem mass spectrometry (LC–MS-MS), for accurate reporting of results (5–7). The specificity of EIAs can be evaluated in terms of cross-reactivity. Although the cross-reactivity of some endogenous and exogenous compounds with EIA kits is usually assessed and listed on the package insert, the process is not exhaustive. The common technique of developing new drugs by modification of the chemical structures of existing drugs further increases the potential for unwanted cross-reactivity with immunoassays (8).

Tapentadol (Nucynta) is a novel centrally acting synthetic analgesic that is indicated for the relief of moderate to severe pain (9–10). It is a Schedule II controlled substance, available in 50, 75 and 100 mg formulations. Tapentadol is believed to have a dual mode of action that enhances its efficacy profile (11–12). The current DRI methadone immunoassay package insert indicates no evaluation of cross-reactivity with tapentadol. Tapentadol (Tap) undergoes extensive biotransformation, primarily through phase II conjugation (70%), forming tapentadol glucuronide (Tap Gluc, 55%) and tapentadol sulfate (Tap Sul, 15%) (9, 13). Metabolism also occurs through phase I oxidative processes (15%), forming *N*-desmethyltapentadol (*N*-DMT, 13%) via CYP2C9 and CYP2C19, and hydroxyl tapentadol via CYP2D6 (2%). Phase I oxidative products undergo further phase II conjugation. Approximately 3% of the urinary product is unchanged tapentadol. As prescriptions for pain management, both tapentadol and methadone are monitored for compliance and abuse with the aid of patient urine drug testing during treatment.

Several pain management urine specimens screened positive and confirmed negative for methadone in the absence of a methadone prescription. Specimens were screened using the DRI methadone immunoassay and confirmed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS-MS). A review of the associated medication lists revealed tapentadol as the only common prescription among the investigated specimens. All specimens screened negative for EDDP using the DRI methadone metabolite immunoassay. The

cross-reactivity of tapentadol and its major metabolites was investigated with the DRI Methadone Enzyme Immunoassay and the DRI Methadone Metabolite Enzyme Immunoassay.

Materials and Methods

Materials

Immunoassay reagents for methadone (reagent lots # 59304905 and #59542128) and methadone metabolite (reagent lots #59307441 and #59457293) were obtained from Microgenics Corp. Methadone control solution for the methadone EIA was purchased from ElSohly Laboratories (Oxford, MS). EDDP control solution for the methadone metabolite EIA was obtained from Microgenics Corp. Negative control solution for both EIAs was acquired from Microgenics Corp. The following compounds were purchased from Cerilliant (Round Rock, TX): tapentadol, *N*-desmethyltapentadol, tapentadol glucuronide, tapentadol sulfate, tapentadol-D3, tapentadol-D3 glucuronide, methadone, EDDP perchlorate, methadone-D3 and EDDP-D3 perchlorate. Certified drug negative normal human urine was obtained from UTAK Laboratories (Valencia, CA).

Methods

Sample analysis

Immunoassay. Enzyme immunoassay analysis of authentic and fortified samples was performed with the DRI immunoassays for methadone and EDDP on an Olympus AU640 analyzer (Beckman Coulter, Irving, TX) according to the protocols described by the manufacturer in package inserts (1–2). Drug-free normal human urine was fortified separately with tapentadol or one of its three major metabolites at various concentrations (100–200,000 ng/mL). Five replicates of each concentration of tapentadol-fortified urine were screened as unknowns alongside methadone and EDDP controls against the DRI methadone and EDDP EIAs, to evaluate cross-reactivity. The controls included a negative control, a positive methadone control (169 ng/mL) and an EDDP control (200 ng/mL). Semi-quantitative EIA results were obtained by quantification with a standard curve of methadone calibrators at 150, 300 and 500 ng/mL (linear range 130–500 ng/mL) purchased from Microgenics. Semi-quantitative EDDP EIA results were obtained by quantification with a standard curve of EDDP calibrators at150, 300 and 1,000 ng/mL(linear range 150–1,000 ng/mL) purchased from Microgenics. Coefficients of variation at the cutoff concentration of 130 ng/mL (n = 30) for the methadone EIA on the Olympus AU640 analyzer did not exceed 5.5%. Coefficients of variation at the cutoff concentration of 150 ng/mL ($n = 30$) for the EDDP EIA on the Olympus AU640 analyzer did not exceed 4.6%.

UPLC–MS-MS. Authentic tapentadol urine specimens that produced false-positive methadone EIA results at a 130-ng/mL cutoff ($n = 97$) were sequestered and stored at 4°C until analysis. For tapentadol confirmation at a 100 ng/mL cutoff, the authentic specimens were diluted forty-fold in water and analyzed on a Waters Acquity UPLC TQD using a modified version of a previously published method (14). The method was updated to include the monitoring of tapentadol glucuronide, tapentadol sulfate, *N*-desmethyltapentadol glucuronide and the internal standard, tapentadol-D3 glucuronide. Samples were not hydrolyzed for tapentadol confirmation, given that the intact conjugates were being monitored. No reference standard was available for *N*-desmethyltapentadol glucuronide, which was determined semi-quantitatively using information from accurate mass determination to deduce transition parameters and *N*-desmethyltapentadol as a reference calibrator (14). Samples also underwent separate methadone and EDDP confirmation at a 50 ng/mL cutoff on a Waters Acquity UPLC TQD using an Acquity UPLC BEH C18 analytical column (1.0 × 50 mm, 1.7 μm). Sample preparation for methadone analysis involved a five-fold dilution in water. Limitations of the UPLC–MS-MS confirmation methods are shown in Table I. Multi-point calibration curves were prepared in normal human urine at the established linear range for each analyte (Table I) and at the same dilution as specimens. Authentic samples with measured concentrations above the upper limit of linearity were repeated at a forty-fold dilution in water relative to the calibration curve. Mass spectral data was acquired in positive electrospray ionization mode with two selected transition ions for all analytes and internal standards, excluding methadone-D3 and EDDP-D3, which were monitored with a single transition ion. Coefficients of variation and percent deviation from target concentrations for all analytes in the UPLC–MS-MS confirmation methods did not exceed 10.9 and 21.7%, respectively.

Data analysis

A positive methadone screen was indicated by a concentration response greater than or equal to the 130 ng/mL cutoff concentration. The percent cross-reactivity was calculated by dividing the resulting positive methadone concentration by the nominal concentration of the test compound in the sample and multiplying by 100 (15).

Results and Discussion

Tapentadol, *N*-desmethyltapentadol, tapentadol glucuronide and tapentadol sulfate all produced positive results for methadone using the methadone EIA kit at specific concentrations in the tested range, indicating cross-reactivity. The cross-reactivity was observed for more than one reagent lot for the methadone EIA. Despite some structural dissimilarity, tapentadol and its metabolites share a phenalkylamine moiety with methadone that may be responsible for the cross-reactivity (Figure 1). The

Table I
Limitations of Confirmation UPLC–MS-MS Methods for Tapentadol and Methadone

Concentration (ng/mL)

Analyte	Limit of detection	Limit of quantification	Upper limit of linearity	Upper limit of carryover
Tapentadol	100	100	500,000	500,000
N-Desmethyltapentadol	100	100	500,000	500,000
Tapentadol glucuronide	25	25	50,000	50,000
Tapentadol sulfate	25	25	50,000	50,000
Methadone	50	50	50,000	50,000
EDDP	50	50	50,000	50,000

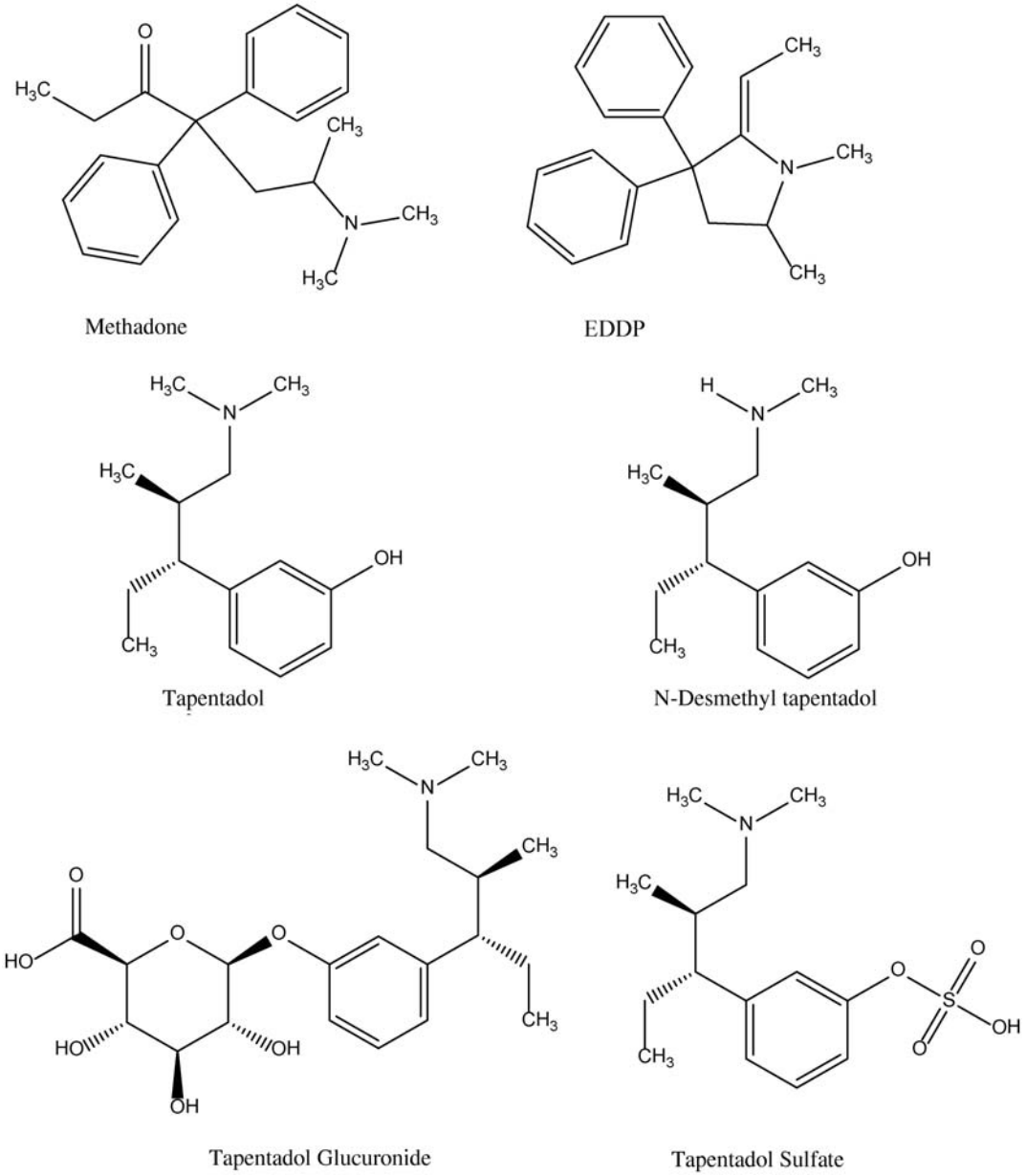

Figure 1. Chemical structures.

common moiety likely allows the tested cross-reacting compounds to fit into active sites in the antibody for the EIA (16). The percent cross-reactivity ranked as follows: tapentadol sulfate > tapentadol > *N*-desmethyltapentadol > tapentadol glucuronide (Table II, Figure 2). The cross-reactivity of tapentadol and its metabolites with the methadone EIA may be influenced by the structural similarity of the molecule to methadone, in terms of molecular weight and the phenalkylamine moiety. Tapentadol sulfate, which exhibited the highest cross-reactivity, is the closest to the methadone structure in this regard. Tapentadol glucuronide is the largest of the analytes and had the lowest cross-reactivity. Its interaction with the methadone EIA may have been limited by steric hindrance. *N*-Desmethyltapentadol exhibited the second lowest cross-reactivity, which may have been due to the slight deviation from the phenalkylamine moiety caused by the loss of a methyl group. Negative results were obtained for tapentadol, *N*-desmethyltapentadol, tapentadol glucuronide and tapentadol sulfate using the methadone metabolite EIA kit at specific concentrations in the range tested, indicating no cross-reactivity. This was not surprising because tapentadol and its metabolites are not structurally similar to EDDP.

All authentic specimens screened positive for methadone with the DRI methadone EIA, registering apparent methadone concentrations ranging from 132–674 ng/mL, but screened negative for EDDP with the DRI methadone metabolite EIA

Table II

Apparent Mean Methadone Concentration (ng/mL) for Samples Fortified with Tapentadol and Metabolites against Methadone EIA Standard Curve*

Standard concentration (ng/mL), $n = 5$	Tapentadol	*N*-Desmethyltapentadol	Tapentadol glucuronide	Tapentadol sulfate
100	3	−1	−7	3
250	8	0	−7	8
500	17	3	−6	22
1,000	37	15	2	53
2,500			16	99
3,000				*131* *4.4%*†
4,000				164
5,000	116	65	38	181
6,000	121			
6,500	*144* *2.2%*†			
7,000	151			
8,000	168			
9,000	184			
10,000	199	109	75	266
15,000		130	102	
20,000		*178* *0.9%*†	123	
25,000	302	210	*161* *0.6%*†	461
50,000	454	268	208	554
100,000	543	418		
200,000	568	536		

*Note: The lowest concentration at which a positive methadone result is triggered is indicated by italics.

†Percent cross-reactivity.

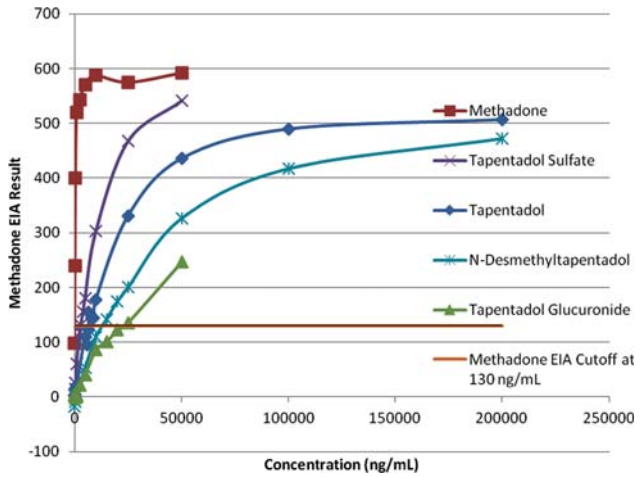

Figure 2. Plot representing mean cross-reactivity of tapentadol and metabolites with DRI Methadone EIA.

(Table III). All authentic urine specimens confirmed negative for methadone and EDDP (Figure 3), but positive for tapentadol and all three monitored metabolites (Figure 4). Concentrations of tapentadol and its metabolites ranged from 121–1,719,133 ng/mL (Table III). As expected, the most abundant urinary excretion product was tapentadol glucuronide, followed by tapentadol sulfate. The relative abundance of unconjugated tapentadol and unconjugated *N*-desmethyltapentadol was

Table III

Summary of EIA and UPLC–MS-MS Results of 97 Urine Specimens from Patients Dosed with Tapentadol

	EIA	UPLC–MS-MS						
	Mtd	Tap	*N*-DMT	Tap-Gluc	Tap-Sul	*N*-DMT Gluc*	Mtd	EDDP
Min (ng/mL)	132	253	121	5744	962	276	0	0
Max (ng/mL)	674	100,663	20,847	1,719,133	268,742	18,694	0	0
Median (ng/mL)	307	7,123	877	106,305	21,162	1,814	0	0
Mean relative abundance (%)	NA	5.4	0.9	76.4	15.8	1.5	NA	NA

*Quantification using *N*-desmethyltapentadol as reference calibrator.

different from predicted values, but consistent with previously reported distributions for authentic samples (14, 17).

Individual analyte concentrations in the authentic samples did not always exceed the analyte-specific cross-reactivity limits, but the associated samples still produced positive methadone EIA results. This provided evidence that combined urinary concentrations of tapentadol and metabolites also contributed an additive cross-reactivity to the methadone EIA. Although the percent cross-reactivity of tapentadol and its metabolites with the EIA kit is low relative to methadone itself, the observed concentrations in authentic specimens corroborate its significance. Total tapentadol concentrations in all authentic specimens exceeded the tapentadol cross-reactivity limit on the methadone EIA, indicating a high frequency at which urinary cross-reactive concentrations may be met. False-positive screening results during routine pain management drug monitoring for methadone are likely in tapentadol prescribed patients when screening with the DRI methadone EIA kit.

Conclusion

Low, but clinically significant, cross-reactivity of tapentadol and its major metabolites was exhibited with the DRI methadone EIA. Microgram concentrations of total tapentadol were detected in authentic tapentadol specimens. The discrimination of such false positives was demonstrated by confirmation analysis using UPLC–MS-MS. The potential is high for false-positive results for methadone EIA screening of urine specimens associated with tapentadol prescriptions. This should be considered when interpreting results, and emphasizes the importance of specific confirmatory testing. The cross-reactivity of tapentadol and its metabolites with other methadone EIA kits should be assessed.

Acknowledgments

The authors wish to thank Evelyn Martin, Georgie Bush and Vicki Robey for assistance in processing samples by EIA screen.

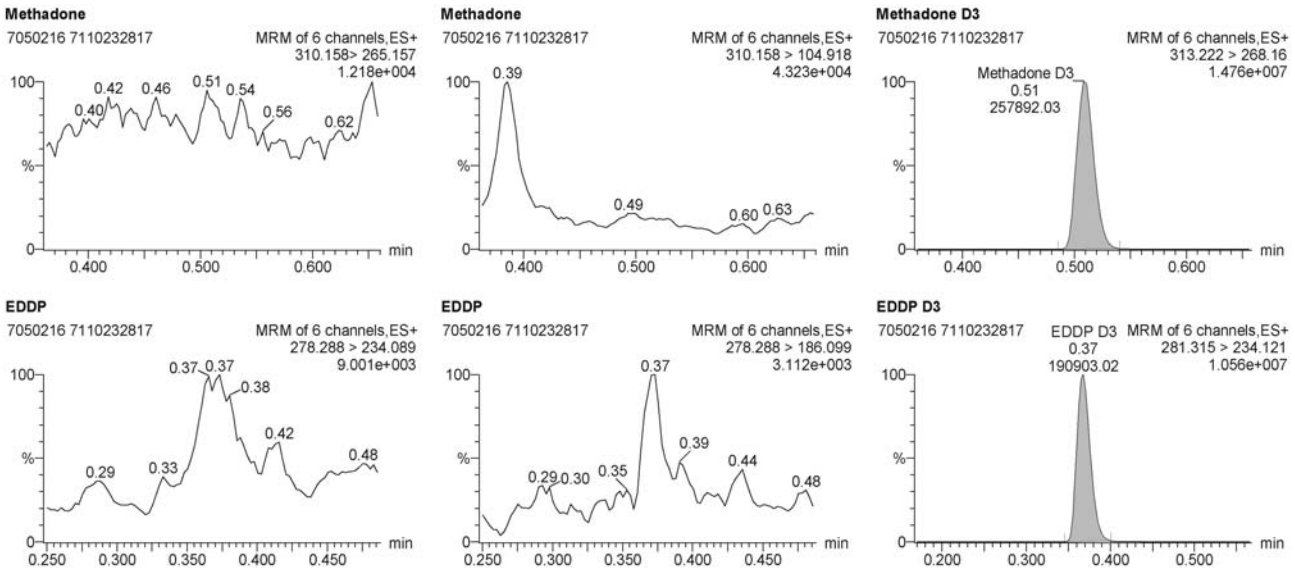

Figure 3. Representative MRM TQD chromatogram for methadone confirmation of an authentic urine specimen TAP2817.

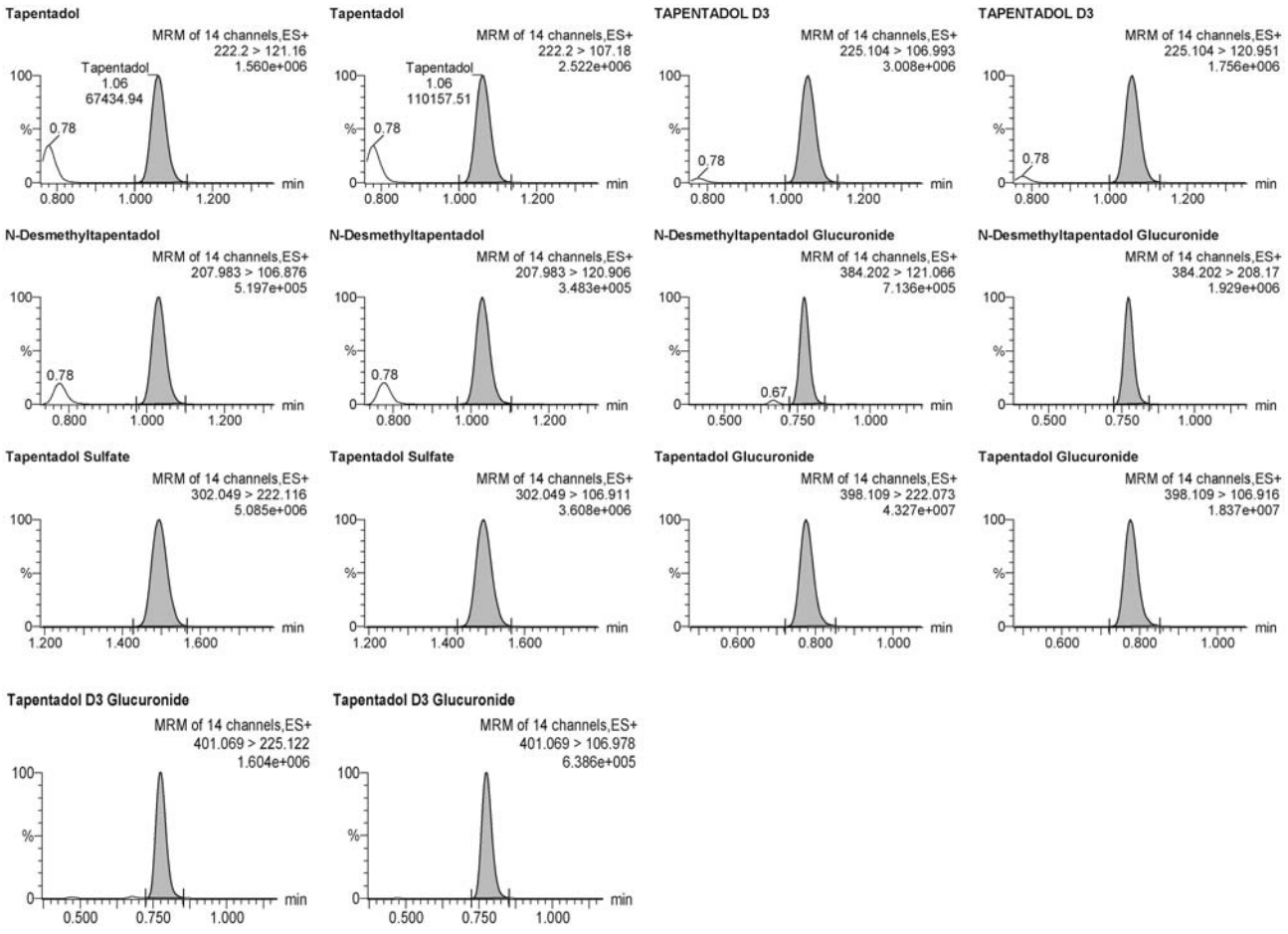

Figure 4. Representative MRM TQD chromatogram for tapentadol confirmation of an authentic urine specimen TAP2817.

References

1. Microgenics DRI. (2003). Methadone Assay package insert. Microgenics Corporation, Fremont, CA
2. Microgenics DRI. (2006). Methadone Metabolite Assay package insert. Microgenics Corporation, Fremont, CA
3. Baselt, R.C. (2004), Disposition of toxic drugs and chemicals in man, 7th edition. Biomedical Publications, Foster City, CA pp. 678–681.
4. Shaikh, S., Hull, M.J., Bishop, K.A., Griggs, D.A., Long, W.H., Nixon, A.L. *et al.* (2008) Effect of tramadol use on three point-of-care and one instrument-based immunoassays for urine buprenorphine. *Journal of Analytical Toxicology*, **32**, 339–343.
5. D'Nicuola, J., Jones, R., Levine, B., Smith, M.L. (1992) Evaluation of six commercial amphetamine and methamphetamine immunoassays for cross-reactivity to phenylpropanolamine and ephedrine in urine. *Journal of Analytical Toxicology*, **16**, 211–213.
6. Logan, B.K., Costantino, A.G., Rieders, E.F., Sanders, D. (2010) Trazodone, meta-chlorophenylpiperazine (an hallucinogenic drug and Trazodone metabolite), and the hallucinogen trifluoromethylphenylpiperazine cross-react with the EMIT® II Ecstasy immunoassay in urine. *Journal of Analytical Toxicology*, **34**, 587–589.
7. Kovatsi, L., Pouliopoulos, A., Papadaki, A., Samanidou, V., Tsoukali, H. (2010) Development and validation of a high-performance liquid chromatography method for evaluation of niflumic acid cross-reactivity of two commercial immunoassays for cannabinoids in urine. *Journal of Analytical Toxicology*, **34**, 229–232.
8. Cody, J.T. (1990) Cross-reactivity of amphetamine analogues with roche abuscreen radioimmunoassay reagents. *Journal of Analytical Toxicology*, **14**, 50–53.
9. (2009) Physicians' Desk Reference, 64th edition. PDR Network, LLC, Montvale, NJ, pp. 2643–2648.
10. Bourland, J.A. (2009) Schedules of controlled substances: Placement of tapentadol into schedule II. Final rule. *Federal Register*, 23790–23793.
11. Christoph, T., De Vry, J., Tzschentke, T.M. (2010) Tapentadol, but not morphine, selectively inhibits disease-related thermal hyperalgesia in a mouse model of diabetic neuropathic pain. *Neuroscience Letters*, **470**, 91–94.
12. Tzschentke, T.M., Jahnel, U., Kogel, B., Christoph, T., Englberger, W., De Vry, J. *et al.* (2009) Tapentadol hydrochloride: A next generation, centrally acting analgesic with two mechanisms of action in a single molecule. *Drugs Today (Barcelona)*, **45**, 483–496.
13. Terlinden, R., Ossig, J., Fliegart, F., Lange, C., Gohler, K. (2007) Absorption, metabolism and excretion of 14C-labeled tapentadol HCl in healthy male subjects. *European Journal of Drug Metabolism and Pharmacokinetics*, **32**, 163–169.
14. Bourland, J.A., Collins, A.A., Chester, S.A., Ramachandran, S., Backer, R.C. (2010) Determination of tapentadol (Nucynta®) and N-desmethyltapentadol in authentic urine specimens by ultra-performance liquid chromatography-tandem mass spectrometry. *Journal of Analytical Toxicology*, **34**, 450–457.
15. Levine, B. (2003) Principles of forensic toxicology, 2nd edition. AACC Press, Washington, DC, p. 136.
16. El-Haj, B., Al-Amri, A., Ali, H. (2008) Letter to the editor: cross-reactivity of nefopam and its metabolites with benzodiazepine EMIT immunoassay. *Journal of Analytical Toxicology*, **32**, 790–792.
17. Coulter, C., Taruc, M., Tuyay, J., Moore, C. (2010) Determination of tapentadol and its metabolite n-desmethyltapentadol in urine and oral fluid by liquid chromatography with tandem mass spectral detection. *Journal of Analytical Toxicology*, **34**, 458–463.