Letter to the Editor

Cross-Reactivity of Nefopam and Its Metabolites with Benzodiazepine EMIT Immunoassay

To the Editor:

Cross-reactivities of some over-the-counter and uncontrolled prescription drugs with the immunoassays of some classes of drugs of abuse have been reported (1–17). Chemical structure similarity is a cause for cross-reactivity in immunoassays because the cross-reacting compound can fit onto the active sites in the antibody that has been developed for the drug of abuse or its chemical class. However, cross-reactivities from compounds that do not show explicit similarities in chemical structures with those of the target analytes are also known (1,7–10,14,15). For enzyme immunoassays, a possible explanation of some of such interferences may lie in enzyme inhibition or enzyme promotion by the interferant (18).

During routine urine screening by EMIT assays (Dade Behring EMIT d.a.u. and EMIT II Plus) for drugs of abuse, we recently detected a positive benzodiazepine test that failed gas chromatographic–mass spectrometric (GC–MS) confirmation after β-glucuronidase hydrolysis and TMS derivatization. However, upon scrutinizing the GC–MS data, the analgesic drug nefopam and its metabolite desmethylnefopam were detected and characterized as the only xenobiotics in the case urine sample. Accordingly, the cross-reactivity of nefopam with the Benzodiazepine EMIT assay was tested using negative-drug urine samples spiked with nefopam in the 5 to 1000 µg/mL concentration range. This was followed by testing urine samples from volunteers who had been given two 60-mg doses of nefopam hydrochloride (Acupan® tablets) separated by a 4-h interval. The results of Benzodiazepine EMIT d.a.u. assays are given in Table I.

Nefopam was found to give a positive Benzodiazepine EMIT assay at a concentration of 25 µg/mL, equivalent to the cutoff value of 200 ng/mL of oxazepam. Although nefopam urine concentrations were not quoted in the three reported overdose fatal cases (19–21), the 25 µg/mL concentration found in this study for Benzodiazepine EMIT assay cross-reactivity is not expected to result in the urine from nefopam overdose. Nevertheless, in the human body, nefopam is metabolized to desmethylnefopam, desmethylnefopam glucuronide conjugate and nefopam-N-oxide (Figure 1). Of the 87% of a dose excreted in the urine, < 5% is unchanged nefopam; metabolites constitute the rest (22). This suggests that the enhanced cross-reactivity of urine from nefopam users with the Benzodiazepine EMIT assay resulted from the contribution of nefopam metabolites. Camara et al. (11) have made the same argument in explaining the cross-reactivity of oxaprozin with a Benzodiazepine immunoassay.

Table I. Benzodiazepine EMIT d.a.u. Readings of Urine Samples of Volunteer Nefopam Users*

Time (h)†	Volunteer I	Volunteer II	Volunteer III
0	165 (–)	154 (–)	159 (–)
3	208 (+)	159 (–)	667 (+)
6	349 (+)	1356 (+)	1866 (+)
12	157 (–)	772 (+)	590 (+)
24	160 (–)	749 (+)	257 (+)
30	154 (–)	149 (–)	161 (–)

* The cutoff value of the assay was 200 ng/mL of oxazepam. All readings above 200 were considered positive for benzodiazepine. The samples were run on an ACE immunoassay analyzer.

† Time: Time interval at which urine sample was collected after start of dosing. *Dosing schedule:* Two 30-mg nefopam hydrochloride (Acupan) tablets at start followed by another two tablets after 4 h.

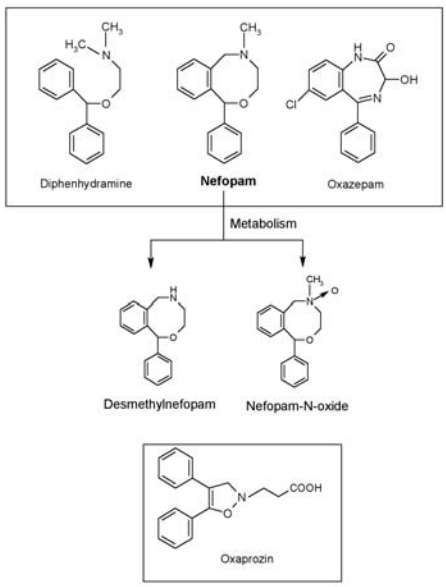

Figure 1. Chemical structures of nefopam and its two metabolites, nefopam open-chain analogue diphenhydramine and the benzodiazepine oxazepam. The chemical structure of oxaprozin is included for comparison purposes. The structures are drawn in such a way as to depict similarities.

The reason for the cross-reactivity of nefopam and its metabolites with oxazepam, in the Benzodiazepine EMIT assay, may be attributed to chemical structural similarity as can be envisaged in Figure 1. Nefopam is a cyclized analogue of the antihistamine diphenhydramine (Figure 1). The cross-reactivity test of the latter drug with the Benzodiazepine EMIT assay was negative at the highest concentration of 1000 µg/mL. This should indicate that the antigenic activity of nefopam and its metabolites for the oxazepam antibody in the immunoassay is a result of the cyclization that results in the benzoxazine-ring system (Figure 1). Furthermore, the increased antigenic property caused by the metabolic demethylation of nefopam may be explained by increased hydrogen-bonding interaction between desmethylnefopam and the antibody; the same reasoning may be extended to the glucuronide conjugate of this metabolite. In fact, hydrogen bonding is one of the intermolecular forces by which antigen-antibody reactions take place (23). It is worth noting that nefopam-N-oxide, though not detected by GC–MS in the case urine samples because of thermal instability, would be expected to cross-react with the Benzodiazepine EMIT immunoassay based on chemical structure similarity.

All of the urine samples in Table I tested positive for nefopam and desmethylnefopam by GC–MS. However, for the three volunteers, the maximum concentration of Nefopam and its metabolites was reached at 6 h post first dose as indicated by the readings obtained for the Benzodiazepine EMIT d.a.u. assay. Positive results were obtained for two of the volunteers 24 h post dosing; by 30 h, samples from all three volunteers were negative. As an analgesic, nefopam is given orally in 30–90-mg doses, three times daily (24). From the results in Table I, 120 mg of nefopam hydrochloride given in two divided doses at a 4-h interval have resulted in false-positive Benzodiazepine EMIT assay tests for at least 24 h. However, in case nefopam is used in pain management for longer periods, a more elaborate study is needed for determining its duration of cross-reactivity with the Benzodiazepine EMIT assay.

In conclusion, in addition to recognizing nefopam and its metabolites as cross-reactants with Benzodiazepine EMIT assay, the present study has emphasized the need for confirmation of immunoassay positive results for drugs of abuse. When a positive immunoassay result of a given class of compounds cannot be confirmed by an alternative method, cross-reactivity should be suspected and potential cross-reactant(s) (as may be identified by chromatographic–MS methods) should be verified by first testing negative urine spiked with the cross-reactant and then carrying out controlled testing of urine from users of the cross-reactant.

Babiker El-Haj and Abdulkader Al-Amri
SHJ Police Forensic Science Laboratory, Forensic Toxicology, Sharjah, United Arab Emirates

Heyam Ali
Dubai Pharmacy College, Pharmaceutical Technology, Dubai, United Arab Emirates

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