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Note

Thin-layer chromatography of benzoylecgonine: a rapid qualitative method for confirming the EMIT Urine Cocaine Metabolite Assays

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The Syva EMIT d.a.u. Cocaine Metabolite Assay is a widely used urine immunoassay for monitoring cocaine (COC) use. It was designed to detect the COC metabolite benzoylecgonine (BE). The assay was considered unsuitable for detecting unchanged COC because of its extremely low cross-reactivity to COC ($<1/625$ of BE) [1]. The confirmation of EMIT-positive responses is usually based on the chromatographic detection of BE [1–3]. Thin-layer chromatography (TLC) is considered an appropriate technique for confirming clinical drug screening assays [4]. Of the TLC methods reported for the detection of BE in urine [5–8] only two [7,8] have BE sensitivities that approach the EMIT low calibrator BE concentration of 0.3 mg/l. An aim of this study was to develop a rugged TLC method able to detect BE at the EMIT low cutoff level. The method developed combined a solid-phase sample preparation first step with a relatively new and rapid method of linear chamber development of a high-performance TLC (HPTLC) silica gel plate.

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It has been proposed [9] and reported [10] that the chromatographic detection of COC or its metabolite ecgonine methyl ester (EME) may serve to confirm an EMIT-positive response. In a large study confirming COC EMIT-positive urines ($n=246$) it was reported [11] that EME and COC were found to a lesser extent (79 and 27% of the samples, respectively) than BE (100% of the samples). Nevertheless, if the physiological concentrations of EME and COC are sufficient to contribute to a positive EMIT response then their detection should be interpreted as confirming the EMIT assay. To estimate the contribution of COC metabolites other than BE to an EMIT-positive response, the assay was tested for cross-reactivity to EME and the EME hydrolysis product ecgonine (EC).

EXPERIMENTAL

Standards, reagents and specimens

COC and COC metabolite standards, solvents and reagents were purchased from commercial suppliers (Applied Science, Deerfield, IL, U.S.A.; Sigma, St. Louis, MO, U.S.A.; Fisher Scientific, Springfield, NJ, U.S.A.). EC was provided by Dr. Richard Hawks of the National Institute on Drug Abuse (Rockville, MD, U.S.A.). Urine specimens were obtained from an in-house drug treatment program and stored at 4°C.

EMIT Urine Cocaine Metabolite Assay

The Syva (Palo Alto, CA, U.S.A.) EMIT d.a.u. and st Urine Cocaine Metabolite Assays were performed according to the manufacturer's protocols. Samples were analyzed by d.a.u. using a Syva AutoLab 6000 system and by st with a Syva QST sample processor. Both processors were connected to a common Gilford Stasar S-III spectrophotometer and Syva data reduction unit. Clinical samples were screened either twice by the d.a.u. assay or once by the d.a.u. and once by the st assay.

TLC confirming assay

Urine aliquots of 3 or 10 ml were taken to confirm samples screened EMIT-positive at or above the EMIT d.a.u. medium 3.0 mg/l or low 0.3 mg/l BE calibrators, respectively. BE was extracted from urine with either a 130-mg (Clean Screen DAU 131) or 300-mg (Clean Screen DAU 303) solid-phase column (World Wide Monitoring, Horsham, PA, U.S.A.). The smaller 130-mg column was used for a 3-ml aliquot and the larger 300-mg column for a 10-ml urine aliquot, respectively. Extractions were processed on a Vac Elut SPS 24 manifold (Analytichem International, Harbor City, CA, U.S.A.). The procedure was as follows: the column was rinsed with one column volume of (1) methanol, (2) water and (3) the acidified urine sample drawn through the column; (4) the column was washed with one column volume of water, 0.1 *M*

hydrochloric acid and methanol; (5) the BE eluted with an aqueous ammonia-saturated mixture of methylene chloride–isopropanol (8:2). The eluate was evaporated to dryness under a stream of nitrogen gas and heat (ca. 45°C). The dry sample residue was dissolved in two 15-μl portions of acetone (6% water) and transferred to a 10 cm×10 cm HPTLC glass-backed silica gel plate (E. Merck No. 5629, Camag Scientific, Wrightsville Beach, NC, U.S.A.) with a glass capillary. The sample extracts were applied in 1-cm streaks to two opposite edges of the plate. Seven samples and one standard containing COC, EME, BE and EC were applied to each side. The chromatogram was developed in a Camag No. 28510 linear developing chamber (Camag Scientific) with ethyl acetate–methanol–methylene chloride–ammonium hydroxide (3:3:1:0.6). After drying with hot air and cooling to room temperature, the plate was sprayed lightly with Ludy Tenger's (L-T) reagent [12] followed by 20% sulfuric acid. BE was visualized as an orange band at R_F=0.25. The R_F values of COC, EME and EC were 0.58, 0.51 and 0.15, respectively. The L-T reagent was prepared by dissolving 0.5 g bismuth(III) carbonate basic (Fluka Chemicals, Ronkonkoma, NY, U.S.A.) in 1.5-ml concentrated hydrochloric acid, adding 3 g potassium iodide and diluting to 50 ml with distilled water. The L-T reagent was stable for up to six weeks when kept at 4°C.

RESULTS

Estimation of extraction column performance

Recovery was estimated by TLC by comparing extracted to unextracted BE areas. Areas were measured by an Amersham Research Analysis System densitometer (Amersham, Arlington Heights, IL, U.S.A.). Aliquots (3 ml) of 3, 2.4, 1.8 and 1.5 mg/l after extraction yielded areas greater than or equal to the corresponding total of unextracted material, suggesting nearly complete BE recovery. The measurement of areas greater than the corresponding total of unextracted material was attributed to a diffuse border between BE and background. The Amersham instrument was capable of resolving only iodoplatinate-visualized BE but not BE visualized with the routine assay's L-T reagent. It was not possible with the Amersham instrument, therefore, to make a direct TLC measurement of the minimum amount of BE visible with the L-T reagent or to determine the recovery of other COC metabolites. However, the column manufacturer states that 94.6±5.4% of BE is recovered at a concentration of 0.05 mg/l.

Evaluation of EMIT Urine Cocaine Metabolite Assay response

The relative reactivity of EME and EC to the EMIT assay were estimated by assaying spiked urine controls. Concentrations of EME and EC less than or equal to 2000 and 100 mg/l, respectively, yielded responses below the EMIT 0.3 mg/l positive cutoff. Representative metabolite concentrations yielding an

EMIT-positive response are shown in Fig. 1. The relative reactivity of EME and EC was estimated at 1/5000 and 1/200 that of BE, respectively. Also shown in the figure is the EMIT-negative response to a urine control mixture of equal volumes of COC, EME and EC at concentrations five to ten times greater than normal [9,13]. Shown too is the effect produced by adding physiological concentrations of COC, EME or EC to an EMIT-negative 0.2 mg/l BE control.

The stability of the EMIT response to BE was evaluated by assaying BE controls over a one-week period (maximum laboratory turnaround time). Urine controls of 0.3, 0.6 and 0.8 mg/l were prepared in negative screened pooled urine ($n=15$). EMIT measurements were made in quintuplicate and the daily means are shown in Fig. 2. Fluctuations were greatest near the 0.3 mg/l cutoff

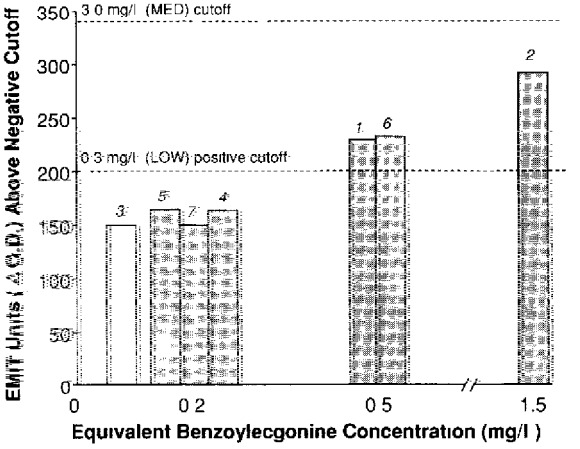

Fig. 1. EMIT d.a.u. reactivity to cocaine metabolites. The abbreviations for cocaine and metabolites are defined in the text. The numbers above each bar in the figure correspond to the following concentrations: (1) 2500 mg/l EME; (2) 300 mg/l EC; (3) equal volume mixture of 50 mg/l COC–500 mg/l EME–50 mg/l EC; (4) 0.2 mg/l BE–12.5 mg/l COC; (5) 0.2 mg/l BE–50 mg/l EME; (6) 0.2 mg/l BE–10 mg/l EC; (7) 0.2 mg/l BE.

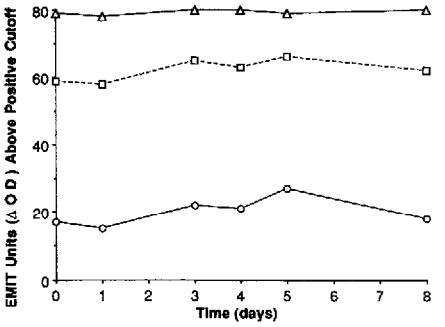

Fig. 2. Stability of EMIT d.a.u.-positive response over a one-week period. (△) 0.8 mg/l BE; (□) 0.6 mg/l BE; (○) 0.3 mg/l BE.

level but stabilized rapidly with increased BE concentration. The EMIT response to the 0.8 mg/l control was unchanged for the week.

Confirmation of EMIT-positives

The TLC confirmation assay was performed on 68 clinical urines screened positive by the EMIT d.a.u. Urine Cocaine Metabolite Assay. The presence of BE was confirmed in all urines screened positive at or above the medium calibrator (43/43) and nearly all those EMIT-positive at or above the low calibrator (19/25). Four of the samples not confirmed were retested four or five days after initial screening. They retested EMIT-negative and thus were presumed negative. The two other unconfirmed samples and a TLC-confirmed sample containing the apparent minimum visible level of BE were analyzed by gas chromatography–mass spectrometry (GC–MS) at a reference laboratory. The concentrations of BE found were 0.04, 0.06 and 0.28 mg/l, respectively.

DISCUSSION

The use of a 10 cm × 10 cm HPTLC plate and linear development shortened chromatographic run time to 10 min and provided excellent metabolite separations and resolution from sample background. A complete confirmation assay was performed in only 30 min. Adequate separations with longer development time also were achieved with conventional TLC on a 20 cm × 20 cm plate and a solvent system of ethyl acetate–methanol–methylene chloride–ammonium hydroxide (3:3:3:0.1). No matter the type of TLC plate or development system used, the application of sample extracts in streaks [14] appeared to prevent overloading and to increase sensitivity by concentrating metabolites into narrow bands. The R_F values of extracted and unextracted COC and metabolites in either development system were identical.

L-T reagent was the chromogenic reagent of choice for visualizing COC metabolites. Like Dragendorff's reagent, it contains bismuth which produces a more intense color reaction to BE than iodoplatinate. Unlike Dragendorff's reagent which produced an orange background with orange to brown COC metabolite bands, L-T reagent produced highly colored red to orange bands on a bright yellow background. The colors intensified with time in the order EC > BE > EME > COC when the reagent was oversprayed with 20% sulfuric acid.

EME did not appear to contribute to the EMIT-positive response. Its relative reactivity was extremely low (1/5000 that of BE). The addition of EME or COC to an EMIT-negative BE control did not change the EMIT response (Fig. 1). However, EC, the EME hydrolysis product, when mixed with an EMIT-negative BE control, increased the EMIT response dramatically. This result was unexpected since the EC and BE controls were each EMIT-negative but together they produced an EMIT-positive response. Thus for EC, mea-

surement of EMIT response to the combination of EC and BE was more reflective of assay activity than measurement of its relative reactivity.

It is possible that an EMIT-positive response could be produced by the combination of a very high level of EC and a level of BE well below the 0.3 mg/l cutoff. It is doubtful that a sample containing this combination would be confirmed by the TLC method. In these cases the presence of BE would need to be confirmed by an alternative GC or GC–MS technique. These conditions might have existed for the two TLC-unconfirmed EMIT-positive samples in which extremely low levels of BE were determined by GC–MS.

Urine controls spiked with BE, BE and EC or BE and EME remained EMIT-positive for at least one week. Four samples screened positive twice by EMIT d.a.u. did not follow the pattern shown in Fig. 2 when retested four or five days later. As a result, the initial screening procedure was changed. The EMIT st test was used in place of the second EMIT d.a.u. test on the assumption that a formulation different from the d.a.u. test might evidence a discrepant response sooner. Two of the four samples were also positive by the st test. When the two were retested four or five days later they were negative by both EMIT tests. The reason for a fleeting EMIT-positive response is not known. Four other samples yielded an initial positive d.a.u. but a negative st response. These four samples also were presumed negative. The changes made to the screening procedure, viz., adding the st test and retesting unconfirmed samples, improved the method for identifying EMIT-negatives. Under the revised EMIT protocol a sample was presumed EMIT-negative if either the initial d.a.u. or st test or their retest within one week was negative.

CONCLUSION

The conclusions of this study are summarized as follows.

(1) The EME metabolite is practically unreactive to the EMIT assay and, therefore, does not contribute to the EMIT-positive response. However, the EME hydrolysis product, EC, does contribute.

(2) A sample containing a BE level below the 0.3 mg/l assay cutoff could produce an EMIT-positive response if approximately 10 mg/l EC were also present in the sample.

(3) A TLC confirmation assay sensitivity equal to the EMIT assay 0.3 mg/l cutoff was sufficient to detect BE in 97% of the EMIT-positives; those not confirmed had BE concentrations approximately 1/5 that of the confirmation and EMIT assay cutoff.

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