was increased substantially in plasma drawn from these porphyric patients at diagnosis [3.04 ± 0.30 and 2.29 ± 0.26 μmol/L for PCT and erythropoietic protoporphyria (EPP) patients, respectively, by direct assay], indicating oxygen-free radical inactivating capacity in these porphyrias (Fig. 1). Specific therapies [34], including chloroquine, β-carotene, charcoal, and cholestyramine, did not interfere. A detailed analysis of the concentrations and their relationship to plasma porphyrin amounts, photosensitivity, and other pathology will be reported elsewhere.

We conclude with the interesting and somewhat chastening observation that nonseparative methods that minimize background absorbance, either through correction factors, or, as in our case, back-extraction into NaOH, allow the MDA-TBA adduct to be measured with specificity comparable with HPLC methods.

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References


acid substitutions [2]. The apo E3 allele is the predominant isoform in all populations studied. The apo E4 allele is associated with increased total serum cholesterol and greater odds for coronary heart disease [3]; it also constitutes a major risk factor for Alzheimer disease [4]. The apo E2 allele seems to have a protective effect against Alzheimer disease and is associated with longevity [5]. Consequently, interest in examining individual patients and study groups for the apo E isoforms is growing. In this communication we describe a simple procedure that facilitates the genotyping of the apo E polymorphisms.

In the common apo E3 polymorphism, TGC codons at positions 112 and 158, whereas in the apo E4 a different CGC codon gives rise to Arg112. The three apo E alleles determine six genotypes, i.e., three homozygotes designated E4/E4, E3/E3, and E2/E2 and three heterozygotes designated E3/E4, E2/E3, and E2/E4.

Early methods for detection of apo E isoforms were based on protein isoelectrofocusing [6]. After the identification of the apo E gene [7] molecular methods based on PCR amplification and HhaI digestion were introduced [8, 9] and later somewhat improved [10, 11]. However, all PCR-based assays are difficult to interpret because the HhaI enzyme yields several small fragments, not all of which are specific for the apo E genotypes. Moreover, incomplete digestion by HhaI can yield ambiguous results. In this study we used two new restriction enzymes, i.e., AfIII and HaeII, that recognize the allele-specific nucleotide substitutions at codons 112 and 158, respectively, and do not recognize additional sites. Fig. 1A illustrates schematically a loss of an AfIII restriction site that is characteristic for the apo E4 allele and a loss of an HaeII restriction site that is unique for the apo E2 allele (see asterisks).

DNA was purified from leukocytes by the salting-out method as described [12]. Genomic DNA was amplified by PCR with the primers F5'-TCCAAGGAGCTGCAGGCGGCGCA and R5'-GCCAAGGCTGGTACACTGCCC to yield a 218-bp DNA fragment that spans both apo E polymorphic sites. In the PCR, 100–200 ng of DNA was added to 25 μL of reaction mixture containing 75 mmol/L Tris-HCl, pH 9.0, 20 mmol/L magnesium sulfate, 0.1 mL Tween, 1.5 mmol/L MgCl₂, 500 mmol/L of each primer, 0.2 mmol/L dNTPs, 100 mL/L dimethyl sulfoxide, and 0.6 units of Taq polymerase (Advance Biotechnology).

The PCR reactions were subjected to 40 cycles in a thermal cycler (MJ Research) with 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 90 s of extension at 70 °C. Amplified DNA (15 μL) was digested simultaneously with 2.5 units of AfIII and 5 units of HaeII (New England Biologicals) for 24 h at 37 °C, analyzed on 4% agarose gel (MetaPhor, FMC), and visualized by ethidium bromide staining.

As expected, simultaneous digestion of the 218-bp amplified product yielded on 4% agarose gel electrophoresis 145-bp, 168-bp, and 195-bp fragments that were specific for apo E3, E2, and E4, respectively (Fig. 1). All six possible genotypes for apo E, i.e., E2/E4, E4/E4, E3/E4, E3/E3, E2/E3, and E2/E2, were clearly discernible (lanes 2–7, respectively). In the E2/E4 genotype (Fig. 1B, lanes 2 and 8) a residual uncut 218-bp fragment was present. To characterize the nature of this uncut fragment, the following experiments were carried out. When the PCR product of the genotype E2/E2 was mixed with the PCR product of the genotype E4/E4 and subjected to simultaneous digestion with AfIII and HaeII, only the bands corresponding to alleles E2 (168 bp) and E4 (195 bp) were observed (Fig. 1B, lane 9). In contrast, when the same mixture of E2/E2 plus E4/E4 was allowed to denature (95 °C for 5 min) and anneal at 55 °C before digestion, the uncut 218-bp band was observed (Fig. 1B, lane 10). These findings were consistent with a heteroduplex formation between DNA strands carrying the E2 and the E4 sequences with its anticipated resistance to the enzyme digestion.

In conclusion, simultaneous digestion of an amplified segment of the apo E gene by AfIII and HaeII enzymes clearly determines all apo E genotypes. The assay is easy...
to perform and can be used for analysis of numerous samples within a short time.

References

Automated On-Line Hydrolysis of Benzodiazepines Improves Sensitivity of Urine Screening by a Homogenous Enzyme Immunoassay, Jeri D. Ropero-Miller, Diana Garside, and Bruce A. Goldberger* (Univ. of Florida College of Med., Dept. of Pathol., Immunol., and Lab. Med., P.O. Box 100275, Gainesville, FL 32610-0275; *author for correspondence: fax 352-846-1586, e-mail goldberg.pathology@mail.health.ufl.edu)

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sant, anxiolytic, anticonvulsant, hypnotic, muscle relaxant, and preanesthetic agents. Moreover, benzodiazepines are often abused for their euphoric effects. Consequently, benzodiazepines are commonly detected in toxicological analyses.

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sant, anxiolytic, anticonvulsant, hypnotic, muscle relaxant, and preanesthetic agents. Moreover, benzodiazepines are often abused for their euphoric effects. Consequently, benzodiazepines are commonly detected in toxicological analyses.

Immunoassays of benzodiazepines are plagued by several factors that make reliable detection difficult. Complicated metabolic pathways, including N-dealkylation, C-hydroxylation, and glucuronidation, effectively reduce the parent compound to more polar, and thus easily excreted, metabolites. Some of these metabolites are pharmacologically active; in some cases, they are present in higher concentrations and have a longer elimination half-life than the parent compound [1, 2]. Many, especially the glucuronide conjugates, which appear at high concentrations in the urine, do not readily cross-react with commercial immunoassays. In contrast, other benzodiazepine metabolites demonstrate high cross-reactivity, depending on the immunoassay utilized [3].

In the US, 13 benzodiazepines of various potencies are commonly prescribed. Therapeutic daily doses have substantially decreased with the newer generation of benzodiazepines [2]. Variations in dosage and in ultimate excretion patterns complicate the ability to accurately detect benzodiazepine use [4].

Accumulated research findings indicate pretreating urine specimens with β-glucuronidase (EC 3.2.1.31) increases the diagnostic sensitivity of benzodiazepine immunoassays. In addition, even untreated specimens with concentrations of benzodiazepine analytes below the cutoff concentration may screen positive because of the presence of highly cross-reactive benzodiazepine metabolites [3, 5]. Also, oxaprozin (Daypro®, G.D. Searle), a structurally unrelated nonsteroidal antiinflammatory drug, produces false-positive results in several commercial immunoassays [6–8].

In this study we evaluated whether automated on-line hydrolysis of benzodiazepine analytes would improve the sensitivity of the Boehringer Mannheim Corp. (BMC) cloned enzyme donor benzodiazepine immunoassay (CEDIA®). We evaluated in parallel 1002 urine specimens that were screened by two CEDIA benzodiazepine assays: the current assay with no enzymatic pretreatment, and a modified assay in which β-glucuronidase (200 U/mL; BMC) was added to the R1 reagent system (5 μL of β-glucuronidase solution per milliliter of R1) for automated on-line hydrolysis of conjugated benzodiazepine analytes. (The modified CEDIA has also been evaluated recently by Bellet et al. [9].) All specimens were analyzed with a Hitachi 717 (BMC) automated chemistry analyzer, with a positive cutoff concentration of 200 μg/L (nitrazepam). All instrument settings were according to published manufacturer’s guidelines for the CEDIA benzodiazepine assay.

All CEDIA presumptive positive specimens (n = 50) were assayed by GC/MS. Before GC/MS analysis, the urine specimens were hydrolyzed with β-glucuronidase (Sigma Chemical Co.), followed by solid-phase extraction (Clean Screen® SPE columns, ZSDAU020; United Chemical Technologies, Horsham, PA). The extracts were derivatized with methyl-(tert-butyldimethylsilyl)-trifluoroacetamide (Pierce Chemical Co.) for quantification of nordiazepam, oxazepam, temazepam, lorazepam, hydroxyethylflurazepam, α-hydroxynitrazepam, and α-hydroxytriazolam. All specimens that were negative by GC/MS were subsequently reextracted and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) for quantification of 7-aminoconazepam, 7-aminoflunitrazepam, and 7-aminoacetazapam.

A Hewlett-Packard (HP) 5890 Series II gas chromatograph and HP 7673 automated liquid sampler interfaced to an HP 5972A mass-selective detector (MSD) were utilized. The gas chromatograph was equipped with a cross-linked 95% dimethyl/5% diphenyl polysiloxane capillary column [HP-5MS; 30 m × 0.25 mm (i.d.) × 0.10-μm-thick film]. Automated injections were made in the splitless mode. The MSD was operated in the selected-ion monitoring mode with