
CLIN. CHEM. 33/5, 697–700 (1987)

Oxybuprocaine and Five Metabolites Simultaneously Determined in Urine by Gas Chromatography and Gas Chromatography-Mass Spectrometry after Extraction with Extrelut®
Fumiyko Kasuya, Kazuo Igarashi, and Miiyoshi Fukui

We describe a gas-liquid chromatographic (GC) method for determination of oxybuprocaine, and a gas chromatograph-mass spectrometric (GC-MS) method for simultaneous determination of four of its nine metabolites in urine. We used an Extrelut® column to simply and rapidly extract oxybuprocaine and its metabolites from urine. For the GC-MS analyses, we monitored the characteristic fragment ions at m/z 353, 395, 369, 411, and 235 for 3-butoxy-4-aminobenzoic acid (metabolite 2, M-2), 3-butoxy-4-aminobenzoic acid (M-3), 3-hydroxy-4-aminobenzoic acid (M-4), 3-hydroxy-4-aminobenzoic acid (M-5), and methaqualone (internal standard), respectively. We quantified the glucuronide of M-2 after enzymic treatment. The assay’s selectivity and reproducibility (within-day and between-day CVs <8% for all metabolites) make it applicable to determine oxybuprocaine and its metabolites in human urine. Mean 9-h urine excretion of oxybuprocaine and its five metabolites from four healthy volunteers was 89.2% after a 100-mg oral dose.

Oxybuprocaine, a local anesthetic, is similar in structure to procaine. Its metabolism in humans is extensive, nine metabolites as well as the parent drug being detectable in urine (1).

Further to elucidate the metabolism of oxybuprocaine, we used GC1 and GC-MS to measure oxybuprocaine and its five principal metabolites in urine specimens from healthy volunteers who had received 100 mg of oxybuprocaine hydrochloride orally.

We also prepared the samples by passing them through 1-mL Extrelut® columns; this extraction generates no emulsions, and so speeds the procedure and provides a high clean-up efficiency (2–6).

Materials and Methods

Reagents: Oxybuprocaine (Santen Pharmaceutical Co. Ltd.) was obtained as the hydrochloride salt. Extrelut® (a wide-pore kieselguhr of grainy structure) was purchased from E. Merck, Darmstadt, F.R.G.; the derivatizing reagent for gas chromatography, \(N,O\)-bis(trimethylsilyl)acetamide (BSA) from Tokyo Kasei, Tokyo, Japan; and methaqualone from Eizai Co., Tokyo, Japan. Other reagents and solvents were of AR grade.

The authentic metabolites (1) of oxybuprocaine and procaine (7, 8) were prepared in the authors’ laboratory.

Instrumentation: For the GC analyses we used a Shimadzu Model GC-6A chromatograph equipped with a hydrogen flame-ionization detector. The 1 m x 3 mm (i.d.) glass column was packed with 2% OV-101 on Chromosorb WHP (100–120 mesh; Hewlett-Packard, Avondale, PA). The column temperature was programmed from 180 to 220 °C at 10 °C/min, then held at 220 °C. The flow rate of the nitrogen carrier gas was 50 mL/min. The injection and the detection temperatures were both 250 °C.

We acquired mass spectra with a Hitachi M-5201 gas chromatograph coupled via a jet separator to a Hitachi M-60 mass spectrometer. The 1 m x 2 mm (i.d.) glass column was also packed with 2% OV-101 on Chromosorb WHP (100–120 mesh). The oven temperature was increased from 160 to 200 °C at 10 °C/min, then held at 200 °C. The injection and the interface temperatures were 230 and 260 °C, respectively. The flow rate of the helium carrier gas was 50 mL/min. The accelerating voltage was 3.0 kV; the ionizing current, 100 μA; and the ionizing potential, 20 eV. The magnet was scanned from m/z 160 to 430 in 8 s, with 1 s allowed for field stabilization between scans. Peak areas were calculated by a computer system, Hitachi 002B Datalizer.

Sample collection: Healthy volunteers were given an oral dose of 100 mg of oxybuprocaine hydrochloride, and urine collected at various intervals up to 9 h after the dose was stored at -20 °C until analyzed.

Pretreatment of the Extrelut column: We used 1-mL Extrelut columns. For assay of the parent drug, we washed the columns with diethyl ether/isopropanol (85/15 by vol). For the acidic metabolites, we loaded 1.0 mL of 0.1 mol/L HCl onto the columns before applying the samples; after 10 min, we eluted the acidic solution retained in the columns by washing with saturated sodium chloride solution. We then washed the columns with methanol, followed by diethyl ether/isopropanol (85/15 by vol).

Eluent solvent: We examined various eluents for use with the Extrelut column. One milliliter of 3-hydroxy-4-aminobenzoic acid solution (100 μg/mL in buffer solution, pH 3) was applied to the Extrelut column. After 15 min, the compound was eluted with 6 mL of each solvent—diethyl ether/isopropanol (85/15 by vol), dichloromethane/isopropanol (85/15), ethyl acetate/isopropanol (80/20), ethyl acetate, chloroform/acetonitrile (95/5), and chloroform/methanol (90/10)—and each solvent was evaporated under reduced pressure. We then determined spectrophotometrically the quantity of 3-hydroxy-4-aminobenzoic acid in each residue by measuring its absorbance at 292 nm in 3 mL of 0.1 mol/L HCl. Analytical recoveries were calculated by comparing the absorbance of each eluate with that of the corresponding aqueous standard.

Extrelut capacity: We assessed the retention capacity of the Extrelut column for each compound by applying 1 mL of 10, 100, 200, 300, and 400 μg/mL solutions of the four metabolites: 3-butoxy-4-aminobenzoic acid (M-2), 3-butoxy-4-acetylaminobenzoic acid (M-3), 3-hydroxy-4-aminobenzoic acid (M-4), and 3-hydroxy-4-acetylaminobenzoic acid (M-5). For elution we used 6 mL of diethyl ether/isopropanol (85/15 by vol). Recovery of each of the synthetic metabolites was determined as described above, except that we measured absorbance at 292 nm for M-2 and M-4, and at 262 nm for M-3 and M-5.

Urine sample preparation of the parent drug: Adjust 1 mL of urine to pH 10 and apply it to the Extrelut column. After 15 min, elute the parent drug with 6 mL of diethyl ether/isopropanol (85/15 by vol) into a 15-mL glass-stoppered tube. To the eluate, add 0.7 mL of the internal standard (1 μg of pramocaine per milliliter in methanol) and evaporate the solvent under reduced pressure. Dissolve the residue in 20 μL of methanol and inject 2-μL aliquots into the GC.

Urine sample preparation of M-2, M-3, M-4, and M-5: Adjust 1 mL of urine to pH 3 and load it on the Extrelut column. After 15 min, elute the metabolites as above. To the eluate, add 0.5 mL of the internal standard (10 μg of methaqualone per milliliter, in methanol). After concentrating the solvent under reduced pressure, transfer the residue to a 2-mL vial and further evaporate the solvent under nitrogen. Dissolve the residue in 0.1 mL of BSA, heat the analyte at 60 °C for 90 min in a heating block, and inject 2-μL aliquots into the GC-MS.

Urine sample preparation for assay of the glucuronide of M-2: To 1 mL of acetate buffer (0.2 mol/L, pH 5.0) and 1 mg of β-glucuronidase (EC 3.2.1.31; 270 Fishman units) in a 15-mL glass-stoppered tube, add 1 mL of urine diluted 1.5- to fivefold with distilled water, and incubate the mixture at 37 °C for 1 h. After centrifuging (700 × g, 15 min), apply 1 mL of the supernatant fluid to the column. The hydrolyzed metabolite is measured by the same methods used to prepare the acidic metabolites before enzyme treatment. To determine the amount of the glucuronide of M-2 that is present, subtract the amount of the metabolite before enzyme treatment from the amount obtained after enzyme treatment.

Quantification: We quantified oxybuprocaine by linear regression of the drug/internal standard peak-height ratio vs concentration (0.1–10 μg/mL). Each acidic metabolite was determined by linear regression of each metabolite/internal standard peak-area ratio vs concentration (0.5–20 μg/mL).

Results and Discussion

Most of the metabolites of oxybuprocaine are phenols, aromatic amines, or carboxylic acids. Because of the mode of isolation of these compounds, in preliminary experiments we examined the effect of pH (range 1–4) on extraction by ethyl acetate of authentic metabolites from aqueous solution. Three metabolites showed maximum recoveries over the pH range 1–3; one metabolite was best extracted at pH 3. Accordingly, we extracted the metabolites from volunteers’ urine at pH 3, then studied the extraction of these compounds retained in the Extrelut columns in further detail.

Extraction efficiency for 3-hydroxy-4-aminobenzoic acid is poorer than for the other compounds. Therefore, we used this as the reference compound in deciding what eluent to use. The highest analytical recoveries were obtained when diethyl ether/isopropanol (85/15) and dichloromethane/isopropanol (85/15) were used as eluents. Recovery after eluting with chloroform/acetonitrile (95/5) or chloroform/methanol (90/10) was only 36 to 38.3%. Guided by these results, we routinely used diethyl ether/isopropanol (85/15) as the column eluent.

The Extrelut columns could retain as much as 400 μg of each acidic metabolite. Even at this high concentration, 6 mL of the eluent eluted 97.5% of the metabolites. Thus 6 mL is a sufficient volume for routine use.

Figure 1 shows gas chromatograms of a standard solution (Figure 1A) and of extracts of human urine obtained by using Extrelut before (Figure 1B) and after (Figure 1C) oral administration of oxybuprocaine hydrochloride. Both oxybuprocaine and the internal standard were well resolved (retention times of 6.7 and 6.5 min, respectively). There were almost no peaks interfering with the drug or the internal standard.

The four acidic metabolites could not be determined by GC because of interference by endogenous substances. Although we attempted some liquid–chromatographic methods, they did not allow simultaneous analysis of the four metabolites. Thus we considered GC-MS with selected ion monitoring, one of the most selective analytical techniques, which is very useful for evaluating trace amounts of materials in complex biological matrices. Through appropriate selection of the ions to be monitored, all of the acidic metabolites could be detected and the background ignored. Chromatographic reproducibility by the GC-MS assay as
Fig. 2. Mass spectra of methaqualone (the internal standard, I.S.) and the TMS-derivatized metabolites of oxybuprocaine described, with use of isothermal temperature, was better than when a temperature program was used. Therefore we used a constant column temperature routinely. Figure 2 shows the mass spectra of methaqualone (the internal standard) and of the trimethylsilyl (TMS) derivatives of the major extractable acid metabolites of oxybuprocaine. Because an acetylamino group as well as a carboxyl and a hydroxyl group were easily derivatized by BSA, the mass spectra of the TMS derivatives of M-3 and M-5 show strong molecular ions at m/z 395 and 411, respectively. The ions at m/z 353, 369, and 235 are also selective and provide the base peaks for the TMS derivatives of M-2 and M-4, and for the internal standard, respectively. Therefore, we performed the analyses by focusing at the characteristic fragment ion of each metabolite as follows: m/z 353, M-2; 395, M-3; 369, M-4; 411, M-5; and 235, the internal standard (Figure 3). The retention times of the TMS derivatives of acidic metabolites (M-2, M-3, M-4, and M-5) were 5.3, 6.0, 3.0, and 3.7 min, respectively, when assayed at a column temperature of 200 °C, and were identical to those of their standard compounds. In blank human urine these monitoring peaks were distinguishable from background and were completely separated from the background peaks.

The correlation coefficients were better than 0.992 for the parent drug, M-3, and M-5, and 0.990 for M-2 and M-4. The calibration curves were linear for each sample over the concentration range studied here.

Mean (±SD) analytical recoveries of 5 µg each of oxybuprocaine, M-2, M-3, M-4, and M-5 added to 1 mL of a control (drug-free) urine were 93.0 ± 1.2%, 92.2 ± 4.3%, 95.3 ± 2.7%, 81.0 ± 3.4%, and 99.2 ± 1.4%, respectively (n = 3).

The precision of the method was assessed at concentrations of 0.2, 0.5, and 1 µg/mL for oxybuprocaine, and 5 and 10 µg/mL for the acidic metabolites. Within-day (n = 5) and between-day (n = 5) CVs for assay of oxybuprocaine ranged from 1.2 to 1.5% and from 1.6 to 3.7%, respectively. The corresponding data for the metabolites were 1.3 to 7.2% and 4.2 to 7.8%.

The present assays are reproducible and specific enough for use in evaluating the metabolism of oxybuprocaine. We
applied the methods to analyses of human urine after oral administration of 100 mg of oxybuprocaine hydrochloride. Figure 4 shows cumulative urinary excretion (% of dose) of oxybuprocaine and its metabolites. Oxybuprocaine is rapidly hydrolyzed to M-2 (9), which is primarily excreted in urine as the glucuronide. The urinary excretion of the drug's metabolites during 9 h was calculated as approximately 83.4%. Only about 6% of the dose was excreted as unconjugated metabolites in urine.

References

CLIN. CHEM. 33/5, 700–704 (1987)

Two Monoclonal-Based Assays for Carcinoembryonic Antigen Compared
Robin A. Felder, R. Hunt MacMillan III, and David E. Bruns

Monoclonal antibody-based methods can provide clinically different impressions for some patients than does an earlier method involving a conventional antiserum. Therefore, performance of two commercial double-monoclonal-antibody methods for carcinoembryonic antigen (CEA) were studied further, those of Abbott Diagnostics and Roche Diagnostic Systems. The two methods showed similar precision. Total CVs over 20 days at three concentrations were 16, 6, and 6% in the Roche assay, and 15, 8, and 7% in the Abbott assay (n = 20). Within-run CVs for quality-control levels 1, 2, and 3, respectively, in the Roche assay were 14, 8, and 9%, and in the Abbott 15, 9, and 10% (n = 20). Mean CEA concentrations were consistently lower in the Roche assay than in the Abbott assay. Results of the two assays correlated significantly in patients with CEA-secreting carcinomas (r = 0.88), and in serial samples from cancer patients who had low, medium, or high CEA concentrations in their sera (r = 0.92 for all serial data). About 10% of values were false-positive (>5.0 µg/L) by each of the two methods in 89 patients with various non-malignant conditions. The two methods appear to be more specific for tumor-associated CEA than are polyclonal antibody-based assays previously available from the two manufacturers.

Carcinoembryonic antigen (CEA), a cell-surface-derived glycoprotein, was first discovered in 1965 (I, 2). Since then, CEA has been widely investigated as a tumor-associated antigen in cases of digestive-tract cancers and other malignant diseases. CEA quantification has been used in monitoring the recurrence or progression of disease, response to therapy, and follow-up management of patients with carcinoma of the colon, breast, lung, prostate, or ovaries (3, 4). However, increased CEA in serum has also been reported in non-malignant inflammatory disorders of the intestinal tract, lung, and liver (5). The assays used in these studies lacked specificity for tumor-related antigen and showed cross reactivity with immunoreactive CEA-like proteins in plasma, such as nonspecific cross-reacting antigen (6), normal fecal antigen-2 (7), biliary glycoprotein-1 (8), and cross-reacting antigen extracted from tumor (9). The increased concentrations of CEA measured in non-malignant diseases, particularly in smokers, were attributed, at least in part, to the nonspecificity of polyclonal antiserum.

The first monoclonal antibodies directed against CEA were developed in 1968 (10, 11), but such monoclonal antibodies have become commercially available only recently. Assays based on monoclonal antibodies purport to have an increased specificity for tumor-related CEA and to yield fewer false-positive results in patients with non-malignant diseases (12). We therefore compared the results of two monoclonal-antibody-based enzyme-linked immunoassay (ELISA) systems for CEA in three patient groups: (a) normal subjects, (b) patients with various non-malignant inflammatory disorders, and (c) patients with CEA-secreting malignancies.

Materials and Methods

Experimental subjects: We measured CEA concentrations in three different subject groups, because non-malignant inflammatory diseases of entodermally derived tissue interfere with the specificity of tumor-associated CEA measurement. We chose 76 non-smoking individuals between the ages of 25 and 70 as the reference population. Eighty-nine patients, ages 15 to 70, with non-malignant inflammatory disorders known to interfere with tumor-associated CEA measurement constituted the second group. The third group consisted of 35 patients, ages 35–80 years, who had CEA-secreting malignant carcinomas, including colorectal carcinoma (94), breast carcinoma (8), cervical cancer (2), esopha-