?Morphine and its analogues ?3.1.Even if the cutoff value of 6– ?acetylmorphine is set to be as low as 0.8 ng/mL, it is being considered necessary to analyze a ?urine specimen sampled not later than 5 h after the heroin intake (Hidetoshi et al., 1970).?o GC/MS conditions; instrument: an HP5890 GC–HP 5989A MS Engine (Agilent ?Technologies, Palo Alto, CA, USA); column (oven) temperature: 200 ?C (0.5 ?min) –>5 ?C/min –>260 ?C (3 min); injection temperature: 200 ?C; injection ?volume: 1 ?L (splitless mode); carrier gas: He; flow pressure: 6 psi; ionization ?mode: EI; electron energy: 70 eV ?3.2.3.Assessment and some comments on the methods ?Qualitative analysis is performed by finding a peak appearing at the same retention time –126 ?Chapter IV ?as that of the authentic standard after trimethylsilyl (TMS) derivatization; it is also important to ?confirm the absence of the corresponding peak in the blank specimen.The residue in the tube is mixed with 50 ?L of N,O–bis(trimethylsilyl)acetamide ?(BSA) reagent, capped airtightly and heated at 80 ?C for 20 min; a 1–?L aliquot of the ?derivatized solution is injected into GC or GC/MS.Quantitation is performed by selected ion monitoring (SIM) using the peak height ?or area ratio of a test compound to IS; the ratio is applied to a calibration curve, which has ?been prepared in advance, to calculate the concentration of the test compound in a ?specimen.After centrifugation at 3,000 rpm for 5 min, the organic (lower) layer is carefully ?transferred to another 50–mL volume glass centrifuge tube of the same type with a ?pipette, followed by the addition of a sufficient amount of anhydrous sodium sulfate ?(2–3 g), and mixed well.The cartridge is washed with 2 mL each of purified water, 0.1 M acetate buffer ?solution (pH 4) and methanol and dried for 2 min by aspiration with a vacuum ?manifold, followed by washing with 3 mL methanol and drying for 5 min again.Since ?morphine and codeine are finally excreted into urine in the conjugated forms with ?glucuronic acid (FUJIMOTO & WAY, 1957; Hidetoshi et al., 1969; Paerregaard, 1957), ?it is necessary to hydrolyze the conjugated forms of these compounds before GC/MS ?analysis.A 2–mL volume of urine and 1.5 mL of concentrated hydrochloric acid are placed ?in a 50–mL volume glass centrifuge tube with a ground–in stopper and heated at 100 ??C for 30 min in a water bath to hydrolyze the conjugated forms of a target ?compound.The target opiate is eluted, by passing 2 mL of dichloromethane/isopropanol/ammonia ?water (80:20:2, v/v) through the cartridge, into a 10–mL volume glass centrifuge ?tube with a ground–in stopper (the shape of the tube bottom preferably to be conical).The poppy seeds are being used ?for various foods; when the foods including the seeds are eaten, an appreciable amounts of ?morphine (including its conjugates) are reported to be excreted into urine (ElSohly et al., ?1990).The organic solution is passed through folded filter paper to remove the dehydrator ?and collected in a 10–mL volume glass centrifuge tube d with a ground–in stopper (?the shape of the tube bottom preferably to be conical).The hydrolyzed solution is mixed with IS solution a, followed by a careful and gradual ?addition of 1.5 mL of 10 M KOH solution saturated with KHCO3 (bubbling appears), ?mixed with 3 mL of 2 M Tris–HCl buffer solution (pH 8.1) and stirred well.However, caution is needed, because a morphine concentration higher ?than the cutoff level can be detected in some urine samples of subjects who have eaten a ?food containing poppy seeds (ElSohly et al., 1990).6– ?Acetylmorphine is alternatively being analyzed as an indicator of heroin abuse; however, even ?with this metabolite, its half–life is only about 40 min, which is much shorter than that of ?unconjugated morphine (3.7 h) (Hidetoshi et al., 1970).The solution is kept at room temperature; ?it is essential to seal

the flask airtightly, because CO2 in atmospheric air can ?be absorbed into the NaOH solution to yield NaHCO3, resulting in the ?decrease of titer of the solution.Low levels (0.1–25 pmol/g tissue) of morphine ?exist in various animals including humans as an endogenous compound; the levels are ?significantly increased by various stresses (Stefano et al., 2000).?o Standard solutions of morphine for a calibration curve (2, 6 and 20 ug/mL): ?a 1–mg aliquot of morphine hydrochloride is dissolved in purified water to ?prepare 1 mg/mL solution; 0.1 mL of this solution is mixed with 4.9 mL ?purified water for 50–fold dilution (20 ug/mL).?o 10 M KOH solution saturated with KHCO3 (100 mL): a 56–g aliquot of KOH ?is dissolved in about 70 mL purified water in a 100–mL volume glass beaker ?with stirring in an ice bath.–123– ?Chapter IV ?o Pretreatment of a solid–phase extraction cartridge (Bond Elut Certify): a 2– ?mL volume of methanol and 2 mL purified water are passed through a Bond ?Elut Certify cartridge just before use.The pH of the solution is confirmed to be 9 with ?a pH test paper; if not, an appropriate amount of the ammonium buffer solution is ?added to it. The solution is extracted with 10 mL of chloroform/ isopropanol (9:1, v/v), ?and the following procedure is performed according to the above iv–vi steps.It should be noted that an amount of ?codeine comparable to that of morphine are also included in the poppy seed– containing foods ?(Hasegawa et al., 1992).Reagents and their preparation ?o Ethylmorphine (internal standard, IS) solution: a 1 mg aliquot of ?ethylmorphine hydrochloride is dissolved in purified water to prepare 1 ?mg/mL solution.For preparing the standard solutions to be used for ?a calibration curve of 6–acetylmorphine hydrochloride or codeine phosphate, –122 ?Chapter IV ?the same dilution procedure is followed.?o 5 M NH4Cl/NH3 buffer solution (pH 9, about 200 mL): a 23.7–mL volume of ?28 % ammonia water solution is dissolved in purified water to prepare 250 ?mL solution (5 M NH3 solution). Analytical conditions ?o GC column: DB–1 (15 m x 0.25 mm i. d., film thickness 0.25 ?m, J & W ?Scientific, Folsom, CA, USA).Procedures ?Two extraction methods, the liquid–liquid extraction and the solid–phase extraction, ?are avail– able; the latter gives cleaner extracts for GC and GC/MS analysis.For quantitative experiments, in addition, a ?0.1–mL volume each of solutions at three concentrations of an opiate (2.6 and 20 ?g/mL) ?is added to 2 mL each of the blank urine; these samples are also processed in the same ?way as above.A 2–mL volume of urine is diluted 2–fold with ?purified water and mixed with 0.1 mL of the IS solution and 0.2 mL of 5 M ?NH4Cl/NH3 buffer solution (pH 9).A 2–mL volume of urine is diluted ?2–fold with purified water and mixed with 0.1 mL of the IS solution and 0.2 mL of ?2 M Tris–HCl buffer solution (pH 8.1).The level of morphine in urine of healthy subjects determined by GC/MS ?was reported to be about 1 ng/mL (Matsubara et al., 1992).Introduction ?There are a number of compounds, such as morphine and codeine, which are ?classified into the opium alkaloids (opiates).They are being used as ethical drugs of narcotic ?analgesics and antitussives; 1 % powder of codeine or dihydrocodeine is commonly ?included in over–the–counter drugs of antitussives. The detection of 6–acetylmorphine is recommendable ?for diagnosis of heroin use, because of its relatively long half–life in the body (Hidetoshi et ?al., 1970). 6–Acetylmorphine hydrochloride ?can be synthesized by the method previously reported (Fehn & Megges, 1985).?o 5 M NaOH solution (100 mL): a 20–g aliquot of NaOH is dissolved in about ?70 mL purified water in a 100 mL volume glass beaker with stirring in an ice ?bath.After the temperature of the solution is cooled to room temperature, it ?is transferred to a 100 mL–volume volumetric flask together with the water

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?solution which has been used for washing the above glass beaker, and the ?volume is adjusted to 100 mL exactly.A 3-mL volume of 5 M NaOH is added to the solution for neutralization, ?followed by the addition of 4 mL of 5 M NH4Cl/NH3 buffer solution (pH 9).The final ?pH of the solution should be checked with a test paper (Whatman, type CF); if the ?pH of the solution shifts from 9, it should be readjusted to pH 9.0 by adding the ?above ammonium buffer solution.A total ion chromatogram (TIC) of the authentic com- ?pounds is shown in Figure IV.7; the mass spectra of the derivatized compounds are shown in ?Figure IV.8.?morphine-TMS: m/z 429 (M+), 414, 401, 287 ?6-acetylmorphine-TMS: m/z 399 (M+), 340, 287, 204 ?codeine-TMS: m/z 371 (M+), 343, 234, 178 ?Ethylmorphine-TMS: m/z 385 (M+) ?nalorphine-TMS: m/z 455 (M+) ?Figure IV.7.The peak of the TMS derivative of ethylmorphine is not included in this chromatogram; but ?it is eluted at 9.4 min under the same conditions.-128 ?Chapter IV ?By SIM of GC/MS, a low level of morphine less than 100 ng/mL can be detected ?and quantitated; however, when a low level of morphine is detected, special care should be ?taken, because of the following reasons.Heroin is rapidly deacetylated and finally excreted into urine as morphine ?glucuronides.?For HPLC analysis of the conjugated forms of opiates, the authentic standards of ?morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) are necessary. A 0.1mL aliquot of this solution is mixed with 1.9 mL of ?purified water for 20-fold dilution (the final concentration in the form of the ?hydrochloride salt: 50 ug/mL), which is stored at -20 ?C.The solution is poured into a Bond Elut Certify Cartridge to let the solution flow ?inside the cartridge slowly taking more than 2 min to adsorb an opiate.A 50-uL volume of BSA is added to 20 ug each of opiates for derivatization, a 0.4-uL aliquot ?each is injected into GC/MS.?Figure IV.6 shows metabolic pathways of morphine, heroin and codeine.Therefore, it is not easy to discriminate the heroin use from morphine use (Cone ?et al., 1991; Hidetoshi et al., 1970).?For accurate diagnosis of a cause of death in an opiate poisoning case, the ratio of a free ?form to a conjugated form becomes important.The latter solution is diluted ?3.33-fold and 10-fold to prepare 6 and 2 ug/mL solutions, respectively, which ?are also stored at -20 ?C.A 13.4-g aliquot of NH4Cl is dissolved in ?purified water to prepare 50 mL solution (5 M NH4Cl solution).The solution is evaporated to ?dryness under a stream of nitrogen with warming the tube at 30-40 ?C.For the unconjugated (free) forms of morphine, codeine and 6-acetylmorphine, ?the extraction is made as follows.If not, the pH of the ?solution is adjusted to 8-9 using the 10 M KOH solution saturated with KHCO3 or 1 M ?HCl solution.?The eluate is evaporated to dryness under a stream of nitrogen with warming at 30-40 ?C. The derivatization and injection into GC or GC/MS are made exactly in the same ?way as that described in the step vi) of the above liquid-liquid extraction section.For the unconjugated (free) forms of morphine, codeine and 6- ?acetylmorphine, the extraction is made as follows.The final ?identification is made by comparing a mass spectrum obtained from a test specimen with that ?obtained from the authentic standard.TIC of TMS derivatives of morphine and its analogues by GC/MS.Mass spectra of TMS derivatives of the authentic morphine and its analogues.A: ?morphine-TMS; B: 6-acetylmorphine-TMS; C: codeine-TMS; D: ethylmorphine-TMS; E: nalorphine- ?TMS.By HPLC, ?the simultaneous analysis of free and conjugated forms is possible without any hydrolysis.Main metabolic pathways of morphine, heroin and codeine.The thick and dash ?arrows show the main and minor metabolic pathways, respectively ?3.2.The volume of the solution is adjusted to about 85 ?mL with

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purified water and left until being cooled to room temperature.Upon saturation, the volume of the solution becomes ?to be about 100 mL. This can be used for experiments and is preservable at ?room temperature.The pH of the solution is confirmed to be 8– ?9; if not, an appropriate amount of the above buffer solution is added to it. The blank ?urine and calibration samples are processed in the same way.The peaks to be detected are as follows (the mass numbers underlined to be used ?for quantitation).Therefore, the detection of ?low levels of morphine starting from a large amount of a specimen cannot be a proof of the ?intake of the drug.Because of these reasons, it seems reasonable to set a cutoff value of morphine in urine ?to be 300 ng/mL.?However, at the present time, GC/MS is being widely used for opiate analysis.An ?appropriate amount of 5 M NH3 solution is mixed with the 5 M NH4Cl ?solution to adjust the pH to 9.0.The ?KHCO3 powder is added to the NaOH solution until its saturation with stirring ?(about 30 g necessary).The above solution is extracted with 15 mL of chloroform/ isopropanol (9:1, v/v) by ?shaking.?The half–life of heroin disappearance in human body was reported to be only about ?2 min.In such a case, an opiate before (free form) ?and after (a total amount) hydrolysis should be analyzed.The amount of a conjugated form ?can be calculated by subtracting the amount of a free form from the total amount.When the flow ?stops, it is aspirated for a moment; then the methanol or water can flow ?through it only by gravity.The purified water should not be passed through ?the cartridge completely; the cartridge should not be dried.As a blank test, 2 mL urine obtained from a ?healthy subject is also treated as above.After cooling to room temperature, 0.1 mL of the IS solution is added to the hydrolyzed ?solution.The blank ?urine and calibration samples are also processed in the same way.These samples are ?equally treated according to the above steps iii–vi.It is, therefore, impossible to detect heroin itself from urine to prove its abuse.GC and GC/MS analysis ?3.2.1.The buffer can be stored at room temperature.For a new cartridge, each solution can ?flow by natural pressure through it without any aspiration.The water flow ?should be stopped when the water volume in the reservoir becomes small.The water level of the water bath should be slightly above the surface ?level of the hydrolysis solution in the tube.–124 ?Chapter IV ?ii.–125– ?Chapter IV ?Solid–phase extraction ?i.The hydrolysis of urine is made according to the step i of the above section.Heroin is not ?derivatized.–121– ?Chapter IV ?Figure IV.6.?Liquid–liquid extraction ?i.?iii.?iv.?vi.?vii.?ii.The pH of ?the final solution is confirmed to be 8–9 with a test paper.?iii.?iv.?3.2.2.?3.2.4.

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