ANALYTICAL METHODS FOR THERAPEUTIC DRUG MONITORING AND TOXICOLOGY

Q. Alan Xu and Timothy L. Madden

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To Hongqin, Michael, and Andy for their love and support.

—QAX

To Mary, Andy, Colleen, and Amy, who help me keep my sanity.

—TLM

CONTENTS

PREFACE

Analytical Methods for Therapeutic Drug Monitoring and Toxicology is a compilation of summarized analytical methods that were previously published in the literature and were designed primarily to serve the needs of pharmacologists, toxicologists, and all other allied health professionals involved in the development, use, or monitoring of pharmaceuticals. The material is presented as structured monographs on 511 different drug entities detailing 964 different analytical methods.

Therapeutic drug monitoring plays a critical role in today's individualized medicine. It consists of an assessment of the clinical indication for performing therapeutic drug monitoring (TDM), chemical analysis of the biological samples, and interpretation of these results for possible therapeutic intervention. Among these, determination of drug concentration in a particular human biological matrix is one of the most critical components of therapeutic drug monitoring. Many analytical procedures have been developed for therapeutic drug monitoring. As analytical techniques and instrumentation have advanced, this area of TDM has probably grown more rapidly than any other. These "growth" techniques have included various types of immunoassay, gas chromatography (GC), high-performance liquid chromatography (HPLC), electrophoresis, and liquid chromatography–mass spectrometry (LC-MS).

Briefly, immunoassay measures a drug molecule as an antigen using a particular antibody, with detection carried out by ultraviolet light absorption, radioactivity, or fluorescence polarization. Immunoassay requires little or no sample preparation and is rapid and easy to use, but a lack of specificity can be problematic with this technique. Electrophoresis has high efficiency in liquid-phase separation and the ability to separate compounds from small inorganic ions to large biomolecules.

Gas chromatography is a useful analytical tool in therapeutic drug monitoring. In this technique drugs are rapidly separated on a GC column heated between 200°C and 350°C. Detectors are the flame ionization detector (FID), the electron-capture detector (ECD), the nitrogen–phosphorus detector (NPD), and the mass spectrometer (MS). Among them, FID and ECD detectors are the most commonly used for drug analysis. The length of capillary columns allows for columns tens of meters long, which provide enormous chromatographic resolving power, but the high temperatures involved necessitate the analysis of thermally stable drugs only.

High-performance liquid chromatography (HPLC) is the major analytical tool used in therapeutic drug monitoring. It is reliable, robust, and dependable. Ultraviolet–visible, fluorescence, refractive index, electrochemical, and photodiode-array detectors are the most frequently used detectors. Because of a large selection of columns and mobile phases, HPLC methods are both versatile and sensitive. Furthermore, the combination of HPLC with mass spectrometry, which is highly sensitive and selective, is becoming the method of choice for therapeutic drug monitoring and toxicology studies.

This book is unique because it contains analytical methods that include not only HPLC methods but also GC, immunoassay, electrophoresis, and newer ultra-high-performance liquid chromatography–ultraviolet (UPLC-UV), and UPLC–tandem mass spectrometry (MS/MS) methods. Our summaries are in detailed text format, providing the reader with a thorough description of method validation. It is intended for use by knowledgeable analysts skilled in analytical techniques such as HPLC, UPLC, GC, LC-MS/MS, GC-MS/MS, immunoassay, and electrophoresis.

Each monograph includes major Chemistry, Method(s), and Reference(s) sections, which include the following items:

Chemistry

1. US adopted name
2. Drug category
3. Chemical name
4. Other names
5. Molecular formula
6. Molecular weight
7. Chemical Abstracts Service (CAS) number
8. Appearance
9. Solubilities
10. pK_a value(s)

Method(s)

11. Individual(s) who developed or published the method
12. Drug that was analyzed
13. Description of analytical system components
14. Description of major operating parameters
15. Standard solution preparation
16. Sample preparation (dilution, liquid–liquid, or solid-phase extraction, derivatization, etc.)
17. Dynamic concentration range of the calibration curve
18. Correlation coefficient and linearity
19. Coefficient of variation of the assay
20. Intraday and interday coefficients of variation in samples
21. Limit of detection and limit of quantification
22. Interference

Reference(s)

23. References

The information in the Chemistry section was obtained from standard reference works [1–3]. In the Method Section, we have included all of the information as completely as possible from the original published articles. Unfortunately, not all of the published analytical methods have included all of the informational items that we think are appropriate for therapeutic drug monitoring and toxicology. Users of this book must also be aware that the presentation of an analytical method in the literature does not exempt them from verifying the adequacy of the method in their own hands. The analyst still must determine fundamental performance parameters of the method, including selectivity, sensitivity, precision, reproducibility, accuracy, limit of detection, and limit of quantification. However, these are more likely to be easily achieved when the method has been demonstrated to work in another laboratory.

The authors of this work acknowledge that there are undoubtedly numerous analytical methods that have been developed for therapeutic drug monitoring and toxicology study; however, space does not allow a complete compilation of all these methods. We have included monographs of drugs that we think are most commonly used.

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Q. ALAN XU AND TIMOTHY L. MADDEN

March, 2010

MONOGRAPHS

ABACAVIR

CHEMISTRY

Abacavir is an antiretroviral. Its chemical name is {(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl] cyclopent-2-enyl}methanol. Other names include Abakavir and Ziagen. Its molecular formula is $C_{14}H_{18}N_6O$, with a molecular weight of 286.3 and a CAS number of 136470-78-5.

METHODS

Assay 1 Dogan-Topal et al. [1] reported an HPLC method for the simultaneous determination of abacavir, efavirenz, and valganciclovir in human serum. An Agilent Technologies 1100 series liquid chromatograph was equipped with a model G1315B diode-array detector (DAD) and model G1329 ALS autosampler. The stationary phase was a Waters Spherisorb column (250 × 4.6 mm, 5 μm particle size). The mobile phase consisted of acetonitrile, methanol, and monobasic potassium phosphate buffer (pH 5.0) (40 : 20 : 40, vol/vol/vol) and was isocratically delivered at 1.0 mL/min. The runtime was 15 min. The injection volume was 20 μL.

Stock solutions of abacavir, efavirenz, valganciclovir, and fluvastatin (internal standard) at 1.0 mg/mL each were prepared in methanol–water (50 : 50, vol/vol). Working solutions were prepared by diluting stock solutions with methanol–water and were stored at −20°C. Standards were prepared by spiking drug-free serum with working solutions. A standard or serum sample (1.0 mL) was mixed with 1.0 mL of acetonitrile, vortexed for 5 min, and centrifuged at 5000 *g* for 10 min. The supernatant was collected and assayed. Retention times for abacavir, efavirenz, valganciclovir, and fluvastatin were 4.1, 11.6, 3.4, and 5.5 min, respectively.

A calibration curve for abacavir was constructed in the concentration range of 50–30,000 ng/mL. The correlation coefficient was 0.999. The coefficient of variation of the assay was 0.3%. Recovery of the drug from serum was better than 98.8%. Limit of detection and limit of quantification were 3.80 and 12.68 ng/mL, respectively. No interference was found from endogenous substances.

Assay 2 Verweij-van Wissen et al. [2] developed an HPLC-UV method for the simultaneous determination of abacavir, didanosine, lamivudine, stavudine, and zidovudine in patient plasma samples. The liquid chromatograph consisted of ThermoElectron model P4000 solvent delivery pump, model AS3000 autosampler, model UV2000 programmable wavelength UV detector, and a ChromJet integrator. The stationary phase was a Waters SymmetryShield RP18 column (150 × 4.6 mm, 3.5 μm particle size) coupled with a SymmetryShield RP18 guard column (20 × 3.9 mm, 3.5 μm particle size). The column temperature was set at 30°C. Solvent A was a mixture of 20 mM potassium acetate buffer (pH 4.60) and acetonitrile (95 : 5, vol/vol), and solvent B consisted of 20 mM potassium acetate buffer (pH 4.60) and acetonitrile (76 : 24, vol/vol). The mobile phase was delivered at 100% A from 0 to 10 min, decreased linearly to 0% from 10 to 24 min, returned to 100% A from 24 to 26 min, and reequilibrated at 100% A for an additional 9 min. The flow rate was 1.0 mL/min. UV detection was performed at 260 nm.

A stock solution of these drugs (0.5 mg/mL each) was prepared in methanol/water (1 : 9). Standards were prepared by fortifying blank human plasma with the stock solution. All solutions were stored at −20°C. Waters Oasis MAX solid-phase extraction (SPE) columns were conditioned with 0.5 mL of methanol followed by 0.25 mL of water. A plasma sample (0.5 mL) and an aliquot (0.5 mL) of water were loaded onto a preconditioned SPE column, drawn through under vacuum, washed with 0.15 mL of water 2 times, dried under vacuum for 5 min, eluted by 0.25 mL of methanol/water (80 : 20, vol/vol) twice, evaporated to dryness at 40°C under a stream of nitrogen, reconstituted in 0.2 mL of acetonitrile/water (5 : 95, vol/vol), vortexed for 20 s, centrifuged for 5 min, and assayed. The injection volume was 25 μL. Under these conditions, retention times for lamivudine, didanosine, stavudine, zidovudine, and abacavir were about 4.3, 5.3, 6.2, 18.7, and 21.3 min, respectively.

Calibration curves for abacavir were linear over the concentration range of 0.015–5 mg/L. Average recovery for abacavir was 101.0%. The accuracy ranged from 97% to 100%. Intraday and interday coefficients of variation were less than 1.9% and 2.3%, respectively. The limit of quantification was 0.015 mg/L.

This assay was free of interference from acetaminophen, acyclovir, amphotericin B, amoxycillin, amprenavir, atovaquone, caffeine, calcium folinate, carbamazepine, clarithromycin, clindamycin, clofazimine, dapsone, domperidone, efavirenz, erythromycin, ethambutol, famotidine, fluconazole, ganciclovir, indinavir, isoniazid, itraconazole, ketoconazole, lidocaine, lopinavir, methadone, nelfinavir, nevirapine, ofloxacin, oxazepam, pentamidine, phenobarbital, phenytoin, pyrazinamide, pyrimethamine, rifabutin, rifampicine, ritonavir, saquinavir, sulfamethoxazole, sulfametrol, tenofovir, trimethoprim, valproic acid, zalcitabine, and its metabolites. Over 1500 patient samples were analyzed using this assay in pharmacokinetic studies.

Assay 3 Aymard et al. [3] developed an HPLC method for simultaneous determination of 12 antiretroviral drugs in human plasma. For the analysis of amprenavir, indinavir, nelfinavir, ritonavir, saquinavir, and efavirenz, a ThermoQuest liquid chromatographic system equipped with an isocratic P1000 pump, AS3000 autosmpler, PC1000 integrator, UV1000 variable-wavelength UV detector, and a Waters column heater was used. The stationary phase was a Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm particles size) protected by a Waters Guard-Pak μBondapak C_{18} precolumn. The column temperature was maintained

Analytical Methods for Therapeutic Drug Monitoring and Toxicology, by Q. Alan Xu and Timothy L. Madden

at 37°C. The mobile phase was composed of 0.04 M dibasic sodium phosphate buffer with 4% (vol/vol) 0.25 M octanesulfonic acid and acetonitrile (50 : 50, vol/vol) and was delivered at 1.3 mL/min. UV detection was carried out at 261 nm between 0 and 9 min, at 241 nm between 9 and 20 min, and at 254 nm between 20 and 32 min. The injection volume was 100 μL.

For the determination of abacavir, didanosine, lamivudine, stavudine, zidovudine, delavirdine, and nevirapine, a liquid chromatographic system consisting of three Beckman model 114M pumps, a Waters model WISP 717 Plus autosampler, a model 481 variable-wavelength UV detector, a Shimadzu RF551 fluorescence monitor, two Lea Switch I&T switch valves, a Cil Cluzeau Croco-Cil column heater, and a Beckman System Gold 2 integrator was used. The stationary phase was a Waters SymmetryShield C_{18} column (250 × 4.6 mm, 5 μm particle size) protected by an UpChurch filter (2 μm). The column temperature was maintained at 30°C. The mobile phase consisted of monobasic potassium phosphate buffer with 1% (vol/vol) 0.25 M octanesulfonic acid and acetonitrile. Mobile phase 1 contained 5% (vol/vol) acetonitrile and was delivered at 1 mL/min from 0 to 12 min, mobile phase 2 contained 20% (vol/vol) acetonitrile and was delivered at 1 mL/min from 12 to 35 min, and mobile phase 3 contained 70% (vol/vol) acetonitrile and was delivered at 1.2 mL/min from 35 to 40 min. UV detection was performed at 260 nm. The fluorescence detector for delavirdine was set at an excitation wavelength of 305 nm and emission wavelength of 425 nm, respectively. The injection volume was 150 μL.

Stock solutions of abacavir, didanosine, lamivudine, and stavudine at 1 mg/mL were prepared in water and stored at 4°C. Stock solutions of zidovudine, delavirdine, efavirenz, amprenavir, indinavir, nelfinavir, ritonavir, and saquinavir at 1 mg/mL and neviapine at 5 mg/mL were prepared in methanol and stored at −20°C. Working solutions of didanosine, stavudine, and zidovudine at 100 μg/mL were prepared by diluting stock solutions with water. Working solutions of saquinavir and efavirenz at 500 μg/mL were prepared by diluting stock solutions with methanol. All working solutions were stored at 4°C. Standards were prepared by spiking drug-free human plasma with working solutions. A plasma sample or standard (1 mL) was loaded onto a J. T. Baker C_{18} extraction column that was preconditioned with 3 mL of methanol followed with 3 mL of distilled water, drawn through the column under pressure, washed with 2 mL of distilled water, dried under vacuum for 1 min, and eluted with 2.6 mL of methanol. The eluate was divided into two aliquots of 1 and 1.6 mL. These two solutions were evaporated to dryness at 40°C under a gentle stream of nitrogen. One residue was reconstituted with 200 μL of water for the analysis of abacavir, didanosine, lamivudine, stavudine, zidovudine, and nevirapine. The other residue was reconstituted with 150 μL of mobile phase for the analysis of amprenavir, indinavir, nelfinavir, ritonavir, saquinavir, and efavirenz. Retention times for indinavir, amprenavir, ritonavir, efavirenz, saquinavir, nelfinavir, lamivudine, didanosine, stavudine, zidovudine, abacavir, and nevirapine were 4.8, 5.6, 12.9, 15.2, 16.8, 29.2, 8.5, 9.6, 11.1, 17.4, 20.9, and 27.9 min, respectively.

A calibration curve for abacavir was constructed in the concentration range of 20–2,000 ng/mL. The correlation coefficient was greater than 0.998. Within-day and between-day coefficients of variation were less than 9.5% and 9.9%, respectively. The limit of quantification was 20 ng/mL. There was no interference with the analysis of amprenavir from the following coadministered drugs except sulpiride: acebutolol, acetaminophen, acetylcysteine, acetylsalicylic acid, acyclovir, albendazole, alimemazine, alizapride, amikacin, amiodarone, amphotericin B, ampicillin, bepridil, buprenorphine, butobarbital, caffeine, calcium folinate, captopril, carbamazepine, carbutamide, chloroquine, ciprofloxacin, clindamycin, clofazimine, clofibrate, clonazepam, clonidine, cloxacillin, clozapine, cocaine, codeine, cortisol, cyamemazine, dantrolene, dexamethasone, dextropropoxyphene, diazepam, diclofenac, digoxin, dihydroergotamine, diltiazem, doxycycline, ethambutol, flecainide, fluconazole, flunitrazepam, fluoxetine, fluvoxamine, foscarvir, furosemide, ganciclovir, gentamicin, glibenclamide, granisetron, halofantrine, haloperidol, imipramine, indomethacin, interferon alfa, isoniazid, itraconazole, josamycin, ketoconazole, levomepromazine, lidocaine, loperamide, loratadine, losartan, mefloquine, meprobamate, methadone, methylprednisolone, metoclopramide, metronidazole, mianserin, moclobemide, morphine, nifedipine, niflumic acid, nitrofurantoin, omeprazole, paroxetine, pentamidine, phenobarbital, phenytoin, piracetam, prazosin, prednisolone, prednisone, primidone, propranolol, quinidine, quinine, ranitidine, ribavirin, rifabutine, rifampicin, roxithromycin, salicylic acid, simvastatin, sulfadiazine, sulfamethoxazole, thalidomide, theophylline, trimethoprim, valproic acid, venlafaxine, vigabatrin, viloxazine, zolpidem, and zopiclone.

More than 500 plasma samples were assayed on each column without significant loss of resolution.

Assay 4 Saux et al. [4] reported the simultaneous determination of abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, and zidovudine in human plasma by high-performance liquid chromatography with tandem mass spectrometry. A ThermoFinnigan Accela liquid chromatograph was coupled with a ThermoFinnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer through a heated-electrospray ionization (HESI) interface. The stationary phase was a Waters Atlantis T3 column (100 × 2.1 mm, 3 μm particle size). The column temperature was maintained at 40°C and autosampler temperature, at 10°C. Solvent A was 0.05% formic acid in water, and solvent B was 0.05% formic acid in methanol. The mobile phase was delivered at 0.250 mL/min at 5% B from 0 to 3 min, linearly increased from 5% to 40% B from 3 to 8 min, and kept at 5% B from 8 to 14 min. The injection volume was 10 μL.

The mass spectrometer was operated in the positive mode: source temperature 50°C, capillary temperature 270°C, capillary voltage 5 kV, nebulizing gas (nitrogen) 35 psi (lb/in.^{2}), auxiliary gas (nitrogen) 30 units, and collision gas (argon) 1.5 mTorr. Abacavir was monitored in the selected-reaction monitoring (SRM) mode: m/z 287.1 → 190.0 at collision energy 20 V and tube lens 120 V. 6-β-Hydroxytheophyline (internal standard) was observed

in SRM mode: m/z 225.1 → 181.3 at collision energy 18 V and tube lens 110 V.

Stock solutions of abacavir and tenofovir at 1000 μg/mL each were prepared in water and didanosine, emtricitabine, lamivudine, stavudine, and zidovudine at 1000 μg/mL in methanol. Working solutions of zidovudine, stavudine, and abacavir (40 μg/mL each), lamivudine, didanosine, and emtricitabine (20 μg/mL each), and tenofovir (10 μg/mL) were prepared by diluting stock solutions with water. A stock solution of 6-β-hydroxytheophyline (internal standard) at 1000 μg/mL was prepared in water and diluted to 2 μg/mL with water. An aliquot (100 μL) of drug-free human plasma was spiked with 50 μL of a working solution, whereas a plasma sample (100 μL) with 50 μL of water, mixed with 50 μL of the internal standard, protein-precipitated with 500 μL of acetonitrile, vortexed for 30 s, allowed to stand at room temperature for 15 min, and centrifuged at 2200 *g* for 10 min at ambient temperature. The supernatant was collected, evaporated to dryness at 35°C under a stream of nitrogen, reconstituted in 500 μL of water, and centrifuged at 2200 *g* for 10 min. The supernatant was assayed. Retention times for abacavir and the internal standard were 8.36 and 8.21 min, respectively.

A calibration curve for abacavir was constructed in the concentration range of 0.020–4.000 μg/mL. The correlation coefficient was 0.995. Within-day and between-day coefficients of vaiation were less than 4.62% and 10.00%, respectively. The recovery ranged from 83.8% to 90.6%.

The observed ion suppression did not influence quantitation of the analytes. No interference was found from endogenous substances or other drugs such as tipranavir, darunavir, fosamprenavir, ritonavir, lopinavir, saquinavir, atazanavir, indinavir, efavirenz, nevirapine, etravirine, enfivirtide, raltegravir, voriconazole, posaconazole, caspofungin, amphotericin B, fluconazole, ceftazidime, ceftriaxone, cefotaxime, ciprofloxacin, perfloxacin, rifampicin, rifabutin, and isoniazid. More than 1000 samples were analyzed for therapeutic drug monitoring using this method.

Assay 5 Notari et al. [5] developed an HPLC-UV assay for the simultaneous analysis of 16 anti-HIV drugs—abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine—in human plasma. A Waters liquid chromatograph consisting of a Waters model 600 pump, model 717 Plus autosampler, and model 2487 UV–visible detector was used. The stationary phase was a Waters Symmetry C_{18} column (250 × 4.6 mm, 5 μm particle size) protected by a Waters Sentry guard column (20 × 3.9 mm) of the same packing material. The mobile phase consisted of 0.01 M monobasic potassium phosphate buffer, and acetonitrile and was delivered at 1.0 mL/min in a gradient mode. The percentage of the phosphate buffer was decreased from 94% to 40% in 10 min, maintained at 40% phosphate buffer for 10 min, further decreased to 0% in 5 min, and returned to 94% phosphate buffer in 10 min. The total runtime was 40 min. UV detections were performed at 240 and 260 nm. The injection volume was 20 μL.

Stock solutions of 16 drugs at 1.0 mg/mL each were individually prepared in methanol. Working solutions were prepared by diluting stock solutions in methanol and were stored at 4°C. Standards were prepared by spiking drug-free human plasma with working solutions. An aliquot of 600 μL of plasma or standard was mixed with 100 μL of methanol, vortexed for 1 min, and centrifuged at 13,000 rpm (rev/min) for 6 min. The supernatant was diluted with 1 mL of water; loaded onto a Waters Oasis HLB cartridge (30 mg/1 mL), which was preconditioned with 1 mL of methanol followed by 1 mL of water; washed with 1 mL of 5% methanol in water; eluted with 550 μL of 0.01 monobasic potassium phosphate buffer, followed by 2 mL of absolute methanol; evaporated to dryness at 36°C under a steam of nitrogen; reconstituted with 100 μL of methanol; and assayed. Under these conditions, retention times (in minutes) were 4.1 (lamivudine), 6.2 (zalcitabine), 7.8 (emtricitabine), 8.6 (didanosine), 9.7 (stavudine), 15.1 (abacavir), 16.2 (zidovudine), 16.6 (nevirapine), 18.1 (indinavir), 19.2 (saquinavir), 19.9 (amprenavir), 21.1 (nelfinavir), 23.1 (ritonavir), 24.5 (lopinavir), 28.4 (efavirenz), and 32.0 (atazanavir).

Calibration curves for abacavir were constructed over the range from 0.025 to 10 μg/mL. Correlation coefficients were 0.9959. The recovery in plasma was 93.6%. The accuracy expressed as the relative percentage error was 8.8%. Intraday and interday coefficients of variation were 8.9% and 9.9%, respectively. The limit of quantification was 0.025 μg/mL. This assay was used routinely at the researchers' institute for therapeutic drug monitoring in HIV-infected patients.

Assay 6 Rezk et al. [6] reported the simultaneous determination of zalcitabine, lamivudine, didanosine, stavudine, zidovudien, abacavir, and nevirapine in human plasma by HPLC with UV detection. An Agilent series 1100 liquid chromatograph consisting of a binary pump, a degasser, an autosampler, and a UV photodiode-array detector was used. The stationary phase was a Waters Polarity dC_{18} column (150 × 3.9 mm, 5 μm particle size) with a guard column (20 × 3.9 mm, 5 μm particle size) of the same packing material. The column temperature was maintained at 40°C. Mobile phase A was 10 mM ammonium acetate buffer adjusted to pH 6.5 with diluted acetic acid. Mobile phase B was a mixture of 200 mL of mobile phase A, 500 mL of acetonitrile, and 300 mL of methanol. The mobile phase was delivered at 1.1 mL/min in a gradient mode. The mobile phase B was delivered at 4% for 15 min, linearly increased to 64% over next 15 min, held at 64% for 3 min, and pumped at 4% for another 7 min. UV detection was performed at 269 nm from 0 to 11 min, at 250 nm from 11 to 14 min, at 271 nm from 14 to 24 min, and at 230 nm from 24 to 33 min. The injection volume was 80 μL.

Stock solutions of zalcitabine, didanosine, and stavudine at 1.0 mg/mL each were separately prepared in water. Stock solutions of lamivudine, abacavir, zidovudine, and nevirapine at 1.0 mg/mL were individually prepared in a mixture of methanol and water (60 : 40). Working solutions were prepared by diluting stock solutions with water. Standards were prepared by spiking human plasma

with working solutions. A stock solution of hexobarbital at 1.0 mg/mL was prepared in acetonitrile. A working solution of hexobarbital at 2.0 μg/mL was prepared by diluting stock solution with 25 mM ammonium acetate buffer (pH 7.0) and was used as an internal standard. An aliquot of 1.0 mL of plasma sample or standard was spiked with internal standard; loaded onto a Varian Bond Elut C_{18} solid-phase extraction cartridge (100 mg/1 mL), which was preconditioned with 1 mL of methanol followed with 1 mL of 100 mM ammonium acetate buffer (pH 7.0); passed through the cartridge; washed with 1 mL of 100 mM ammonium acetate buffer (pH 7.0); dried under vacuum for 1 min, eluted with 800 μL of methanol; evaporated to dryness at 40°C under a stream of nitrogen; reconstituted with 100 μL of mobile phase; vortexed for 30 s; and centrifuged at 18,000 *g* for 3 min. The supernatant was separated and assayed. Under these conditions, retention times for zalcitabine, lamivudine, didanosine, stavudine, zidovudine, abacavir, nevirapine, and hexobarbital were 5.9, 8.6, 13.6, 15.7, 23.8, 25.1, 27.3, and 30.6 min, respectively.

Calibration curves for abacavir were constructed over the range from 10 to 10,000 ng/mL. Correlation coefficients were greater than 0.998. The accuracy was 101%. Intraassay and interassay coefficients of variation were less than 7% and 7.6%, respectively. The limit of quantification was 10 ng/mL. There were no interferences from either endogenous compounds in plasma or drugs such as indinavir, amprenavir, saquinavir, nelfinavir, ritonavir, lopinavir, delavirdine, efavirenz, tenofovir, and atorvastatin.

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ABECARNIL

CHEMISTRY

Abecarnil is a β-carboline compound and is studied as an anxiolytic and anticonvulsant. Its chemical name is isopropyl 6-(benzyloxy)-4-(methoxymethyl)-9*H*-pyrido(3,4-b)indole-3-carboxylate. Other names include Abecarnilo and ZK112119. Its molecular formula is $C_{24}H_{24}N_2O_4$, with a molecular weight of 404.5 and a CAS number of 111841-85-1.

METHOD

Assay 1 Krause et al. [1, 2] described the determination of abecarnil by HPLC with fluorescence detection. A liquid chromatographic system was composed of a Waters model 6000A pump, model 710B WISP autosampler, and a Kratos model MS970 fluorescence detector. The stationary phase was a Spherisorb ODS II column (125 × 4.6 mm, 5 μm particle size). The mobile phase consisted of methanol and 0.01 M ammonium carbonate buffer (70 : 30, vol/vol) and was isocratically delivered at 1.5 mL/min. Fluorescence detector was set at an excitation wavelength of 295 nm and an emission wavelength of 418 nm. The injection volume was 200 μL.

5-Benzyloxyabecarnil was used as an internal standard. Plasma or urine (0.5 mL) was mixed with 2.5 mL of diethylether, shaken for 15 min, centrifuged at room temperature at 1500 *g* for 10 min, and frozen. The organic phase (top layer) was collected, evaporated to dryness under a stream of nitrogen, reconstituted with 250 μL of the mobile phase, and assayed.

A linear calibration curve for abecarnil was constructed in the concentration range of 0.1–200 ng/injection. The recovery of the drug from plasma was about 90%. The between-day (interday) coefficient of variation was less than 11%. The limit of detection was 0.2–0.5 ng/mL. No interference was found from endogenous substances.

A similar method was used by Karara et al. [3].

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ACAMPROSATE CALCIUM

CHEMISTRY

Acamprosate calcium is used in treatment of alcohol dependence. Its chemical name is calcium 3-acetamido-1-propanesulfate. Other names include Acamprosatum

Calcicum, Campral, and Zulex. Its molecular formula is $C_{10}H_{20}CaN_2O_8S_2$, with a molecular weight of 400.5 and a CAS number of 77337-73-6. Acamprosate calcium is a white powder. Acamprosate calcium is freely soluble in water and practically insoluble in ethanol and dichloromethane.

METHODS

Assay 1 Luo et al. [1] developed a LC-MS/MS method for the determination of acamprosate calcium in human plasma. A Shimazdu 10AVP liquid chromatograph included model LC10ADVP pump, model SIL-HTc autosampler, and model CTO10ASVP column oven. The stationary phase was a Phenomenex Gemini C_{18} column (50 × 3.0 mm, 3 μm particle size). The column temperature was maintained at 50°C. The mobile phase consisted of 10 mM ammonium acetate and methanol (95 : 5), adjusted to pH 7.4 with ammonia. The flow rate was 0.2 mL/min.

An API3000 tandem mass spectrometer coupled to the liquid chromatograph was operated in a negative electrospray ionization mode. Acamprosate calcium was monitored in multiple reaction monitoring mode: m/z 180 → 80. The operating parameters were as follows: nebulizer gas 13, curtain gas 10, core energy −55 V, capillary energy −3.6 kV, collision energy −32 V, and source temperature 450°C.

Acamprosate calcium stock solution 200 mg/L was prepared in water and stored at 4°C. Working solutions were prepared by diluting the stock solution with water. Standards were prepared by spiking the blank human plasma with working solutions. Patient plasma or standards (150 μL) were mixed with 500 μL of acetonitrile and centrifuged at 16°C at 10,000 rpm for 8 min. Supernatants (500 μL) were collected, evaporated to dryness at 50°C in a water bath under a stream of air, reconstituted in 100 μL of the mobile phase, mixed with 1.0 mL of dichloromethane, and centrifuged at 16°C at 2000 rpm for 3 min. Supernatants were collected and assayed. The injection volume was 15 μL. Under these conditions, the retention time of acamprosate calcium was 2.4 min.

A calibration curve for acamprosate calcium was constructed in the range from 2 to 2048 μg/L. The correlation coefficient was 0.9999. Within-day and between-day coefficients of variation were less than 4.0% and 11.6%, respectively. Recoveries of acaprosate calcium in plasma ranged from 83.6% to 94.4%. The limit of detection was 2.0 μg/L.

Assay 2 Girault et al. [2] described the determination of acamprosate calcium (calcium acetylhomotaurinate) in human plasma and urine by gas chromatography–mass spectrometry (GC-MS). A Hewlett-Packard 5985B gas chromatograph–mass spectrometer was utilized. The injection port was maintained at 320°C. The oven temperature was programmed from 240°C to 310°C at 10°C/min and held at 310°C for additional 2 min. Helium was used as the carrier gas. The stationary phase was a Chrompack fused-silica capillary column (25 m × 0.35 mm) wall-coated with an OV1701 liquid phase. The film thickness and inner side diameter of the capillary column were 0.2 μm and 0.25 mm, respectively.

The mass spectrometer was set in the negative-ion chemical ionization mode and operated at an electron energy of 100 eV, an emission current of 300 μA, and an ion-source temperature of 150°C. 4-Acetylaminobutane sulfonic acid was used as an internal standard. The drug and internal standard were monitored in a single-ion monitoring (SIM) mode: m/z = 424 and 317 for the pentafluorobenzoyl di-*n*-butylamide derivative of acamprosate calcium and m/z = 438 and 311 for the derivative of internal standard.

Stock solutions of acamprosate calcium at 1 μg/mL and internal standard at 10 μg/mL were individually prepared in water and stored in the dark at 4°C. Standards in plasma were prepared by spiking 1 mL of the blank human plasma with 50 μL of internal standard and desired amouts of the stock solution of the drug. Standards in urine were prepared by fortifying 50 μL of blank human urine with 50 μL of internal standard and various amounts of the drug stock solution. The drug was extracted from plasma and urine and derivatized as described below. A plasma sample or standard (1 mL) was mixed with 1 mL of acetonitrile, vortexed for 20 s, and centrifuged at 1600 *g* for 15 min. The supernatant was collected, extracted with 5 mL of methylene chloride for 10 min, and centrifuged for 15 min. The aqueous layer was removed and hydrolyzed with 0.2 mL of 10 M hydrochloric acid at 100°C for 2 h. A urine sample or standard (50 μL) was hydrolyzed directly. The hydrolyzed solution was applied to a J. T. Baker C_{18} disposable solid-phase extraction cartridge that was preconditioned with 1 mL of methanol followed by 1 mL of distilled water. The eluate was collected and alkalized with 5 M sodium hydroxide solution. This solution was mixed with 20 μL of pentafluorobenzoyl chloride, adjusted to pH 3 with 2 M hydrochloric acid, washed twice with 3 mL diethyl ether, and centrifuged. The aqueous layer was collected, mixed with 0.1 mL of 10% tetrabutylammonium hydrogensulfate solution and 5 mL of methylene chloride at room temperature for 10 min, and centrifuged for 10 min. The organic layer was collected, evaporated to dryness at 45°C under a gentle stream of nitrogen, redisolved in 50 μL of thionyl chloride, incubated at 80°C for 30 min, mixed with 0.2 mL of 2 M di-*n*-butylamine in acetonitrile, acidified with 1 mL of 20% phosphoric acid, extracted with 4 mL of pentane, and centrifuged. The organic layer was collected, evaporated to dryness under a stream of nitrogen, reconstituted in 0.3 mL of ethyl acetate, and assayed. The injection volume was 1 μL. Under these conditions, retention times of acamprosate calcium and the internal standard were 5.5 and 6.2 min, respectively.

A calibration curve for acamprosate calcium was constructed daily in the concentration range of 3.12–800 ng/mL. The correlation coefficient was greater than 0.9999. The coefficient of variation of the assay was less than 7.9%. There was no interference with the analysis of the drug from endogenous compounds. This method was applied to the pharmacokinetic study of acamprosate calcium in 24 healthy volunteers.

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ACEBUTOLOL HYDROCHLORIDE

CHEMISTRY

Acebutolol is a cardioselective β-blocker. Its chemical name is (±)-3′-acetyl-4′-(2-hydroxy-3-isopropylaminopropoxy)-butyranilide hydrochloride. Other names include Abutol, Prent, and Sectral. Its molecular formula is $C_{18}H_{28}N_2O_4 \cdot HCl$, with a molecular weight of 372.9 and a CAS number of 34381-68-5. Acebutolol hydrochloride is a white or almost white crystalline powder. Acebutolol hydrochloride has solubilities of 200 mg/mL in water and 70 mg/mL in alcohol. It is very slightly soluble in acetone and dichloromethane and practically insoluble in ether. Acebutolol has an apparent pK_a of 9.4 in water.

METHODS

Assay 1 Umezawa et al. [1] described the simultaneous determination of four ß-blockers, acebutolol, labetalol, metoprolol, and propranolol in human plasma, using LC-MS/MS. An Agilent 1100 series system consisting of a model G1315A diode-array detector, model G1313A autosampler, and model G1322A vacuum membrane degasser was utilized. The stationary phase was a Shodex MSpak GF310 4B column (50 × 4.6 mm, 6 μm particle size) protected by a 2-μm SUMIPAX PG-ODS inline filter. Solvent A was 10 mM ammonium acetate aqueous solution and solvent B, acetonitrile. The mobile phase was delivered at 0.55 mL/min in a gradient mode at 100% A from 0 to 3 min, decreased to 0% A in 1 min, and kept at 0% A from 4 to 9.5 min. The column was reequilibrated at 100% A for 5.5 min.

An Applied Biosystems/MDS SCIEX API2000 triple quadrupole mass spectrometer equipped with a TurboIonSpray ion source was operated in the positive mode: TurboIonSpray temperature 490°C, ion source voltage 3 kV, ring voltage 390 V, nebulizer gas (high-purity air) 20 psi, heater gas (high-purity air) 80 psi, curtain gas (high-purity nitrogen) 40 psi, orifice voltage 41 V for acebutolol, 25 V for labetalol, 40 V for metoprolol, 51 V for propranolol, and 40 V for pindolol, collision gas (nitrogen) 4, collision energy −29 eV for acebutolol, −22 eV for labetalol, −25 eV for metoprolol, −25 eV for propranolol, and −25 eV for pindolol. Quantification was performed in selective-reaction monitoring (SRM) mode using ion transitions at m/z 337 → 116 for acebutolol, m/z 329 → 311 for labetalol, m/z 268 → 116 for metoprolol, m/z 260 → 116 for propranolol, and m/z 249 → 116 for pindolol, respectively.

Stock solutions of acebutolol, labetalol, metoprolol, propranolol, and pindolol (as internal standard) at 1 mg/mL were separately prepared in methanol. Working solutions were prepared by diluting stock solutions with 10 mM ammonium acetate in water. Calibrators were prepared by spiking drug-free plasma with working solution. An aliquot of 1 mL of a plasma sample or calibrator was mixed with 3 mL of 13.3 mM ammonium acetate aqueous solution and centrifuged at 9000 rpm for 10 min. The supernatant was filtered through a 0.2-μm Whatman GD/X syringe filter (13 mm) and assayed. The injection volume was 100 μL. Under these conditions, retention times of labetalol, metoprolol, acebutolol, propranolol, and pindolol were 6.6, 6.9, 7.2, 7.8, and 7.9 min, respectively.

Calibration curves for acebutolol were constructed in the range from 10 to 1000 ng/mL. Correlation coefficients were greater than 0.9992. The effect of ion suppression on the analysis of acebutolol was less than 29.8%. The recovery of acebutolol from plasma ranged from 74.4 to 89.9%. The accuracy ranged from 94.0% to 111%. Intraday and interday coefficients of variation were less than 2.9% and 5.6%, respectively. The limit of detection was 1 ng/mL.

Assay 2 Vieno et al. [2] developed an LC-MS/MS method for the detection of acebutolol, atenolol, metoprolol, sotalol, carbamazepine, ciprofloxacin, ofloxacin, and norfloxacin in drinking water, surface water, and sewage treatment plant water. An Agilent 1100 series system consisting of a binary pump, vacuum degasser, autosampler, and a thermostated column oven was used. The stationary phase was an Agilent Zorbax XDB C_{18} column (50 × 2.1 mm, 5 μm particle size) protected by an Agilent narrowbore guard column (12.5 × 2.1 mm, 5 μm particle size). The column temperature was maintained at 30°C. The mobile phase consisted of acetonitrile and 1% acetic acid in water and was delivered at 250 μL/min in a gradient mode from 3% to 28% acetonitrile in 12 min and to 53% acetonitrile in another 5 min, kept at 53% acetonitrile for 1 min, and then returned to the initial condition in 1 min. The column was equilibrated at 3% acetonitrile for 8 min.

A Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization interface was operated in positive mode: desolvation gas 640 L/h, nebulizing gas 30 L/h, collision gas (argon) 2.8×10^{-3} mbar, source temperature 120°C, and desolvation temperature 325°C. Cone voltage (V) and collision energy (eV) were 28 and 20 for acebutolol, 30 and 23 for atenololol, 25 and 15 for metoprolol, 30 and 23 for sotalol, 25 and 15 for alprenolol (internal standard), 29 and 18 for carbamazepine, 35 and 21 for dihydro-carbamazepine (internal standard), 30 and 17 for ciprofloxacin, 28 and 16 for norfloxacin, 29 and 18 for ofloxacin, and 28 and 18 for enrofloxacin (internal standard). Quantification was performed in multiple-reaction monitoring (MRM) mode using ion transitions at m/z 336.8 → 116.0 for acebutolol, m/z 267.0 → 144.9 for atenolol, m/z 267.9 → 190.9 for metoprolol, m/z 254.8 → 132.9 for sotalol, m/z 249.9 → 172.9 for alprenolol, m/z 237.0 → 193.9 for carbamazepine, m/z 239.0 → 193.9 for dihydrocarbamazepine, m/z 331.9 → 287.9 for ciprofloxacin, m/z 319.8 → 275.9 for norfloxacin,

m/z 361.8 → 317.9 for ofloxacin, and m/z 359.9 → 315.9 for enrofloxacin, respectively.

Stock solutions of drugs were prepared in methanol, except that antibiotics were prepared in a mixture of methanol and 0.01 M hydrochloric acid (1 : 1, vol/vol) and stored at −18°C. Working solutions were prepared daily by diluting stock solutions with the same solvents. Standards were prepared by spiking noncontaminated groundwater with working solutions and internal standards. A sample (100, 250, 500, and 1000 mL for sewage influent, sewage effluent, surface water, and groundwater, respectively) was adjusted to pH 10.0 with 2 M sodium hydroxide solution, spiked with 500 ng of the internal standards, and filtered through a 0.45 μm Schleicher & Schuell GF6 filter that was previously washed with *n*-hexane, acetone, methanol, and water. It was then loaded onto a Waters Oasis HLB solid-phase extraction cartridge (3 mL, 60 mg) by means of polytetrafluoroethylene (PTFE) (Teflon) tubes at flow rates of 2, 5, 10, and 20 mL/min (sewage influent water, sewage effluent water, surface water, and groundwater, respectively), which was preconditioned sequentially with 2 mL of *n*-hexane, 2 mL of acetone, 10 mL of methanol, and 10 mL of noncontaminated groundwater (pH adjusted to 10.0); pulled through the cartridge; washed with 2 mL of 5% methanol in 2% aqueous ammonium hydroxide; dried with a stream of nitrogen for 30 min; eluted with 4 × 1 mL of methanol; evaporated to near dryness under a stream of nitrogen; reconstituted with 20 μL of methanol and 480 μL of 1% acetic acid; and assayed. Under these conditions, retention times of sotalol, atenolol, norfloxacin, ofloxacin, ciprofloxacin, enrofloxacin, acebutolol, metoprolol, alprenolol, carbamazepine, and dihydrocarbamazepine were 3.3, 4.4, 9.8, 9.8, 10.2, 10.9, 11.1, 11.2, 15.4, 17.6, and 17.8 min, respectively.

Calibration curves for acebutolol were constructed in the range from 0.82 to 6000 μg/L. Correlation coefficients were greater than 0.996. Average recoveries of acebutolol from groundwater and tapwater, surface water, sewage effluent water, and sewage influent water were 93%, 105%, 78%, and 64%, respectively. Limits of quantification in drinking (potable) water, surface water, sewage effluent water, and sewage influent water were 0.4, 0.8, 2.1, and 6.4 ng/L, respectively.

This LC-MS/MS method was successfully applied for the determination of acebutolol in sewage influent water, sewage effluent water, and their recipient rivers.

Assay 3 Delamoye et al. [3] developed an HPLC method for simultaneous determination of 13 ß-blockers and one metabolite: atenolol, sotalol, diacetolol, carteolol, nadolol, pindolol, acebutolol, metoprolol, celiprolol, oxprenolol, labetalol, propranolol, tertatolol, and betaxolol. A Spectra liquid chromatographic system consisting of a model P1000XR quaternary gradient pump, model AS3000 autoinjector with a 100-μL loop, and model 6000LP photodiode-array detector was used. The stationary phase was a ThermoHypersil Hypurity C_{18} column (250 × 4.6 mm, 5 μm particle size) protected by a C_{18} precolumn (4 × 4.4 mm, 5 μm particle size). The column temperature was maintained at 35°C. UV detection was performed at 220 nm. The injection volume was 80 μL.

Stock solutions of these compounds at 1.0 g/L each were prepared in methanol. Working solutions were prepared by diluting stock solutions with methanol. A stock solution of medroxalol at 50.0 mg/L in methlanol was used as an internal standard. These stock and working solutions were stored at −20°C. An aliquot of 1 mL of plasma, standard, or control was spiked with 20 μL of the internal standard, mixed with 500 μL of 1 M sodium carbonate (pH 9.7), extracted with 7 mL of chloroform–pentanol–diethyl ether (6 : 2 : 1, vol/vol/vol), shaken for 15 min, and centrifuged at 3000 *g* for 5 min. The organic phase was collected, mixed with 250 μL of 0.05 M phosphoric acid (pH 2.1), shaken for 10 min, and centrifuged at 3000 *g* for 5 min. The aqueous phase was collected and assayed. Under these conditions, retention times for atenolol, sotalol, diacetolol, carteolol, nadolol, pindolol, acebutolol, metoprolol, celiprolol, oxprenolol, medroxalol, labetalol, propranolol, tertatolol, and betaxolol were 5.1, 5.6, 7.9, 9.2, 9.9, 10.5, 14.5, 15.4, 18.8, 20.4, 21.2, 21.8, 24.6, 25.1, and 25.9 min, respectively.

Calibration curves for acebutolol were constructed over the range from 25 to 1000 ng/mL. The mean correlation coefficient was 0.999. The mean accuracy was 100.1% at 100 ng/mL. The mean recovery ranged from 90 to 113%. Intraday and interday coefficients of variation were 6.27% and 6.60%, respectively. Limits of detection and quantification were 6 and 25 ng/mL, respectively.

Assay 4 Lee et al. [4] developed an LC-MS/MS method for the simultaneous determination of 12 ß-blockers and $ß_2$-agonists in sewage samples. A Waters 2695 separation module was used. The stationary phase was an Agilent Zorbax SB C_8 column (150 × 2.1 mm, 3.5 μm particle size) protected by a SB C_8 guard column(12.5 × 2.1 mm, 5 μm particle size). The column temperature was maintained at 35°C. Mobile phase A was a mixture of water, acetonitrile, and formic acid (94.5 : 5.0 : 0.5, vol/vol/vol), and mobile phase B was a mixture of acetonitrile and formic acid (99.5 : 0.5, vol/vol). The mobile phase was delivered in a gradient mode from 100% A to 75% A in 13 min, held at 75% A for 13 min, and then pumped at 100% A for another 14 min. The flow rate was 0.2 mL/min. The injection volume was 10 μL. The total runtime of an injection was 40 min.

A Micromass Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray ionization source was operated in the positive-ion mode. The major parameters were set as follows: nebulizer gas (nitrogen) 50 L/h, desolvation gas (nitrogen) 500 L/h, source temperature 120°C, desolvation temperature 350°C, capillary voltage 3.45 kV, cone energy 60 V, and collision energy 17 kV. Acebutolol was analyzed in the multiple-reaction monitoring (MRM) mode at ion transitions m/z 337 → 116 for quantitation and m/z 337 → 260 for confirmation.

Individual stock solutions of acebutolol, alprenolol, atenolol, bisoprolol, clenbuterol, fenoterol, labetalol, metoprolol, nadolol, pindolol, propranolol, sotalol, terbutaline, and timolol at 1000 μg/mL were prepared in acetonitrile or methanol. A stock solution of salbutamol was prepared at 500 μg/mL. These stock solutions were stored at −20°C. Working solutions were prepared by mixing and diluting

these stock solutions with mobile phase B. An aliquot of 250 mL of sewage sample was filtered through a 1.2-μm GF/C filter (90 mm i.d.) with a layer of Celite; acidified to pH 3 with 1 M hydrochloric acid; loaded onto a Waters Oasis MCX cartridge (6 mL, 150 mg, 30 μm) at a flow rate of 10–15 mL/min, which was preconditioned with 6 mL of methanol followed by 10 mL of water at pH 3; dried for 10 min under vacuum; washed with 100 mL of water at pH 3 followed by 6 mL of methanol; eluted with 8 mL of a mixture of dichloromethane, 2-propanol, and ammonium hydroxide (78 : 20 : 2, vol/vol/vol); evaporated to dryness at 40°C under a stream of nitrogen; reconstituted in 1.0 mL of mobile phase B; filtered through a 0.45-μm nylon syringe filter; and assayed. Under these conditions, retention times for acebutolol, alprenolol, atenolol, bisoprolol, labetalol, metoprolol, nadolol, pindolol, propranolol, sotalol, timolol, clenbuterol, fenoterol, salbutamol, and terbutaline were 17.36, 24.32, 8.96, 21.77, 22.11, 18.02, 13.60, 14.15, 23.87, 7.85, 17.02, 17.91, 11.50, 6.97, and 6.53 min, respectively.

Calibration curves for acebutolol were constructed over the range from 50 to 500 pg/μL. The mean recovery of the drug from water ranged from 88% to 95%. The limit of detection was 9 ng/L.

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ACECLOFENAC

CHEMISTRY

Aceclofenac is a NSAID (nonsteroidal anti-inflammatory drug). Its chemical name is [*o*-(2,6-dichloroanilino) phenyl]acetate glycolic acid ester. Other names include Aceclofar, Aceclofenaco, Aceclofenacum, Beofenac, and Preservex. Its molecular formula is $C_{16}H_{13}Cl_2NO_4$, with a molecular weight of 354.2 and a CAS number of 89796-99-6. Aceclofenac occurs as a white or almost white crystalline powder. Aceclofenac is practically insoluble in water. It is soluble in alcohol and freely soluble in acetone. Aceclofenac should be stored in airtight containers and protected from light.

METHODS

Assay 1 Jin et al. [1] reported an HPLC method for the determination of aceclofenac in human plasma. A Shimadzu LC10A system equipped with a model SPD10A UV detector was used. The stationary phase was a Shimadzu ODS column (150 × 4.6 mm, 5 μm particle size). The mobile phase consisted of methanol and 0.1 M ammonium acetate aqueous solution (pH 6.0) (7 : 3, vol/vol) and was isocratically delivered at 1.0 mL/min. UV detection was performed at 275 nm and 0.005 AUFS (absorbance units full scale). The injection volume was 20 μL.

An aliquot of 0.5 mL of a plasma sample was spiked with 75 μL of 1 M hydrochloric acid, vortexed, mixed with 3.5 mL of ether, shaken for 3 min, and centrifuged at 3000 rpm for 10 min. An aliquot of 3 mL of the supernatant was collected, evaporated to dryness at 37°C under a stream of nitrogen, reconstituted with 0.2 mL of mobile phase, and assayed. Under these conditions, the retention time of aceclofenac was about 6.9–8.4 min.

A calibration curve for aceclofenac was constructed in the concentration range from 0.05 to 40.0 mg/L. The correlation coefficient was 0.9999. The average recovery of aceclofenac from plasma was 82.5%. Intraday and interday coefficients of variation were less than 7.1% and 9.3%, respectively. The limit of quantification was 0.05 mg/L. The analysis of aceclofenac was not affected by endogenous compounds in plasma.

Assay 2 Lee et al. [2] described the simultaneous determination of aceclofenac and diclofenac in human plasma by narrowbore HPLC using a column-switching technique. A Shiseido Nanospace SI-1 series liquid chromatograph consisting of two model 2001 pumps, model 2002 UV–visible detector, model 2003 autosampler, model 2004 column oven, model 2012 high-pressure switching valve, and model 2009 degassing unit was utilized. The stationary phase was a Phenomenex Luna 2 phenylhexyl narrowbore column (100 × 2 mm, 3 μm particle size). The column temperature was maintained at 30°C. The mobile phase consisted of acetonitrile and 0.02 M potassium phosphate buffer (pH 7) (33 : 67, vol/vol) and was delivered at 0.2 mL/min. UV detection was performed at 278 nm.

Stock solutions of aceclofenac and diclofenac at 1 mg/mL were prepared in methanol. Standards were prepared by spiking drug-free human plasma with stock solutions. An aliquot of 100 μL of a plasma sample was filtered through a 0.2-μm membrane filter and introduced onto a Capcell Pak MF Ph1 precolumn (20 × 4 mm) to remove proteins using a mixture of acetonitrile and 0.1 M potassium phosphate buffer (pH 7) (14 : 86, vol/vol) at 0.5 mL/min from 0 to 6.0 min. The valve was switched to a Capcell Pak C_{18} UG120 column (35 × 2 mm) to concentrate drugs from 6.0 to 8.8 min. From 8.8 to 17 min, the valve was switched to the analytical column to separate drugs using the mobile phase at 0.2 mL/min. Under these conditions, retention times of diclofenac and aceclofenac were about 13 and 14.3 min, respectively (estimated from the published chromatogram).

A calibration curve for aceclofenac was constructed in the range from 50 to 10,000 ng/mL. The correlation

coefficient was 0.999. The mean recovery of aceclofenac from plasma was 90.5%. Intraday and interday coefficients of variation were 2.8% and 3.0%, respectively. The limit of detection was 10 ng/mL.

Assay 3 Zinellu et al. [3] evaluated a capillary electrophoresis method for the simultaneous determination of aceclofenac and diclofenac in human plasma. A Beckman MDQ capillary electrophoresis system equipped with diode-array detector was utilized. The uncoated fused-silica capillary had 75 μm i.d. and 40 cm in length (30 cm to the detection window). The injection was made at 3.5 kPa for 15 s under vacuum, and the injection volume was 112 nL. The run buffer was 300 mM sodium borate aqueous solution containing 200 mM *N*-methyl-D-glucamine, adjusted to pH 8.9 with 5 M sodium hydroxide solution. The separation of drugs was carried out at 30 kV (140 μA), 25°C, and normal polarity. UV detection was performed at 290 nm.

A plasma was mixed with an equal volume of 200 μL of acetonitrile, vortexed, centrifuged at 3000 *g* for 5 min, and directly injected onto the capillary. Under these conditions, migration times of aceclofenac and diclofenac were about 2.8 and 3.1 min, respectively.

Calibration curves for acecelofenac were constructed in the range from 2.5 to 40 mg/L. Correlation coefficients were greater than 0.999. Intraday and interday coefficients of variation were 4.2% and 5.8%, respectively. Limits of detection and quantification were 0.03 and 0.1 mg/L, respectively.

Assay 4 Hinz et al. [4] reported the simultaneous analysis of aceclofenac and its metabolites in human plasma by HPLC. A liquid chromatographic system equipped with a Jasco model PU980 gradient pump and a Spectra Physics model Spectra 100 detector was used. The stationary phase was a Machery–Nagel Nucleosil 120-5 C_{18} column protected by a C_{18} precolumn. The column temperature was maintained at 30°C. Solvent A was a mixture of 0.005 M phosphate buffer and acetonitrile (20 : 80, vol/vol) and solvent B, a mixture of 0.01 M phosphate buffer and acetonitrile (88 : 12, vol/vol), where 0.01 M phosphate buffer was prepared by dissolving 7.1 g of dibasic sodium phosphate dodecahydrate and 6.8 g of monobasic potassium phosphate in 5 L of distilled water. The mobile phase was delivered at 1 mL/min in a gradient mode as follows:

Time (min)	%A	%B
0	4	96
24	4	96
25	12	88
40	12	88
41	15	85
60	15	85
61	4	96
76	4	96

UV detection was performed at 282 nm. The injection volume was 100 μL.

Stock solutions of aceclofenac and its metabolites, diclofenac, 4′-hydroxyaceclofenac, and 4′-hydroxydiclofenac, were prepared in acetonitrile and stored at −80°C. Standards were prepared by spiking blank human plasma with stock solutions. Ketoprofen at 0.05 mg/mL in acetonitrile − 0.01 M phosphate buffer solution (pH 2.5) (95 : 5, vol/vol) was used as an internal standard. An aliquot of 1 mL of a plasma sample or standard was mixed with 0.5 mL of 40 mg/mL sodium fluoride solution and 0.1 mL internal standard, extracted with 5 mL of *n*-hexane/diethylether (50 : 50, vol/vol), shaken for 30 min, and centrifuged at 4000 rpm for 10 min. The organic layer was collected; evaporated to dryness under a stream of nitrogen; reconstituted in 120 μL of a mixture (pH 2.5) containing 72% 0.01 M phosphate buffer, 15% acetonitrile, 10% methanol, and 3% tetrahydrofuran; and assayed. Under these conditions, retention times of aceclofenac, diclofenac, 4′-hydroxy-aceclofenac, 4′-hydroxy-diclofenac, and ketoprofen were 69.1, 60.9, 46.9, 28.4, and 21.2 min, respectively.

Linear relationships were observed over the concentration ranges from 10 to 10,000 ng/mL for aceclofenac, 4′-hydroxyaceclofenac, and diclofenac and from 25 to 10,000 ng/mL for 4′-hydroxydiclofenac. Correlation coefficients were greater than 0.996. Intraday coefficients of variation were less than 9.9%, 10.9%, 6.3%, and 6.3% for aceclofenac, 4′-hydroxyaceclofenac, diclofenac, and 4′-hydroxydiclofenac, respectively. Interday coefficients of variation were less than 2.7%, 9.3%, 4.2%, and 5.6% for aceclofenac, 4′-hydroxyaceclofenac, diclofenac, and 4′-hydroxydiclofenac, respectively.

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ACEMETACIN

CHEMISTRY

Acemetacin is a NSAID. Its chemical name is *O*-[(1-*p*-chlorobenzoyl-5-methoxy-2-methylindol-3-yl)acetyl] glycolic acid. Other names include Acemetacinum, Bayf-4975, Emflex, Rantudil, and TVX-1322. Its molecular formula is $C_{21}H_{18}ClNO_6$, with a molecular weight of 415.8 and a CAS number of 53164-05-9.

METHODS

Assay 1 Shi et al. [1] reported an HPLC method for simultaneous determination of acemetacin and its metabolite, indomethacin, in human plasma. A Shimadzu liquid chromatographic system consisted of a model SPD10AD pump, model SPD10A UV detector, model SIL10A autosampler, model CBM10A integrator, and a Sys-tee column heater. The stationary phase was a Kromasil C_{18} column (150 × 4.6 mm, 5 μm particle size) protected by a frit (0.5 μm). The column temperature was maintained at 30°C. The mobile phase consisted of 0.02 M monobasic sodium phosphate buffer (pH 4.5), acetonitrile, and methanol (400 : 300 : 300) and was isocratically delivered at 1.0 mL/min. UV detection was carried out at 254 nm. The injection volume was 40 μL.

Stock solutions of acemetacin and indomethacin at 1000 μg/mL were prepared in methanol and stored at 4°C. Working solutions were prepared by diluting stock solutions with 50% methanol in water. Flurbiprofen at 1000 μg/mL in methanol was used as an internal standard and stored at 4°C. Standards of acemetacin and indomethacin were prepared by fortifying the blank human plasma with working solutions. A standard or plasma sample (0.5 mL) was mixed with 0.5 mL of 0.02 M monobasic sodium phosphate buffer; vortexed for 1 min, centrifuged at 10,000 rpm for 10 s; loaded onto a Varian Bond Elut C_2 disposable cartridge (100 mg, 1 mL) that was preconditioned sequentially with 1 mL of mobile phase, 1 mL × 2 of methanol, and 1 mL of water; drawn through the cartridge under vacuum; washed with 1 mL of 0.02 M monobasic sodium phosphate buffer and then with 0.1 mL of mobile phase; dried under vacuum for 5 min; eluted with 0.25 mL of mobile phase; mixed with 25 μL of internal standard (4 μg/mL in mobile phase); vortexed for 1 min, and assayed. Retention times for acemetacin, flurbiprofen, and indomethacin were 5.88, 7.10, and 9.19 min, respectively. There was no interference from endogeneous compounds in plasma.

A calibration curve for acemetacin was constructed in the concentration range of 20–1000 ng/mL. The correlation coefficient for acemetacin was 0.9990. The recovery of acemetacin from plasma ranged from 89.5% to 91.5%. Intraassay and interassay coefficients of variation for acemetacin were less than 3.9% and 4.7%, respectively. The limit of detection was 20 ng/mL.

A calibration curve for indomethacin was also obtained in the concentration range of 20–1000 ng/mL. The correlation coefficient was 0.9980. The recovery of indomethacin from plasma ranged from 73.2% to 76.9%. Intraassay and interassay coefficients of variation for indomethacin were 7.3% and 9.0%, respectively. The limit of detection was 20 ng/mL.

Assay 2 Ban et al. [2] developed an HPLC method for the pharmacokinetic study of acemetacin in human plasma. A Waters 2690 Alliance separation module equipped with a Waters 2487 (dual-wavelength) absorbance detector was used. The stationary phase was a Shiseido Capcell Pak C_{18} reversed-phase column (150 × 4.6 mm, 5 μm particle size). The mobile phase consisted of 20 mM potassium phosphate buffer (pH 2.9) and acetonitrile (60 : 40, vol/vol) and was isocratically delivered at 1 mL/min. UV detection was performed at 254 nm. The injection volume was 80 μL.

Stock solutions of acemetacin, its metabolite (indometacin), and flurbiprofen (internal standard) at 10 mg/mL were prepared in methanol. Working solutions were prepared by diluting stock solutions with mobile phase. Stock solutions were stored at −20°C. Standards were prepared by spiking blank human plasma with working solutions. An aliquot of 1 mL of a plasma sample or standard was spiked with 20 μL of the internal standard at 25 μg/mL, mixed with 1 mL of 100 mM potassium phosphate buffer (pH 2.0), extracted with 9 mL of ethyl acetate, vortexed for 5 s, and centrifuged at 3000 *g* for 10 min. The organic layer was collected, evaporated to dryness under a stream of nitrogen at 30°C, reconstituted with 125 μL of mobile phase, and assayed. Under these conditions, retention times of flurbiprofen, indometacin, and acemetacin were 21.0, 25.1, and 27.3 min, respectively.

Linear calibration curves for acemetacin and indometacin were constructed over the range from 100 to 400 ng/mL. Correlation coefficients were 0.9998 for acemetacin and 0.9999 for indometacin. The average accuracy for acemetacin ranged from 98.1% to 107.5%. Intraday and interday coefficients of variation for acemetacin were less than 16.6% and 12.3%, respectively. The average accuracy for indometacin ranged from 97.7% to 102.5%. Intraday and interday coefficients of variation were less than 15.8% and 19.5%, respectively. Limit of quantification was 100 ng/mL for both compounds.

Assay 3 Hu et al. [3] described the determination of acemetacin and indometacin in human serum by HPLC. A Shimadzu system equipped with a model LC10A pump, model SPD6AS UV detector, and model CR2AS integrator was utilized. The stationary phase was a Spherisorb C_8 column (250 × 4.6 mm, 5 μm particle size). The mobile phase consisted of acetate buffer (pH 4.6), methanol, and acetonitrile (55 : 5 : 40, vol/vol/vol) and was isocratically delivered at 1.0 mL/min. UV detection was performed at 254 nm and 0.005 AUFS. The injection volume was 20 μL.

Standards were prepared in blank human serum, and the final concentraton of tolbutamide as internal standard was 0.5 mg/L. An aliquot of 0.4 mL of a plasma sample or standard was spiked with 20 μL of the internal standard, mixed with 0.2 mL of acetate buffer (pH 4.6), vortexed for 10 s, extracted with 3 mL of diethyl ether, vortexed for 2 min, and centrifuged at 4000 rpm for 10 min. A portion of 5 mL of the organic layer was collected, evaporated to dryness at 45°C under a stream of nitrogen, reconstituted with 0.2 mL of mobile phase, and assayed. Under these conditions, retention times of acemetacin, indometacin, and tolbutamide were 5.5, 7.5, and 8.8 min, respetively.

Linear calibration curves for acemetacin were constructed over the range from 12.5 μg/L to 1.6 mg/L. Correlation coefficients were greater than 0.9996. Intraday and interday coefficients of variation were 3.6% and 5.6%, respectively. The average recovery was 78.3%. Limits of detection and quantification were 0.25 ng and 6.2 μg/L, respectively.

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ACENOCOUMAROL

CHEMISTRY

Acenocoumarol is an oral anticoagulant. Its chemical name is (*RS*)-4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl] coumarin. Other names include Acenocumarin, Acenocumarol, G-23350, Sinthrome, and Sintrom. Its molecular formula is $C_{19}H_{15}NO_6$, with a molecular weight of 353.3 and a CAS number of 152-72-7. Acenocoumarol occurs as an almost white to buff-colored odorless or almost odorless powder. It is practically insoluble in water and ether and slightly soluble in alcohol and chloroform. The drug dissolves in aqueous solutions of alkali hydroxides.

METHOD

Assay 1 Rentsch et al. [1] reported a normal-phase HPLC assay for the stereospecific determination of *R*- and *S*-acenocoumarol and *R*- and *S*-phenprocoumon in human plasma. A Varian liquid chromatograph consisting of a model 9010 pump, model 9100 autosampler with a 100-μL loop, and model 9050 UV–visible detector was utilized. The stationary phase was a Merck LiChroCART S,S-Whelk-01 chiral column (250 × 4.0 mm, 5 μm particle size) protected by a Merck LiChrosper 100 DIOL guard column (4 × 4 mm, 5 μm particle size). Eluent A was a mixture of *n*-hexane and ethanol (90 : 10, vol/vol) containing 0.5% acetic acid, and eluent B was a mixture of *n*-hexane and ethanol (60 : 40, vol/vol) containing 0.5% acetic acid. The mobile phase was delivered at 1.0 mL/min from 5% B to 50% B in 20 min, increased to 100% B in another 5 min, and was maintained at 100% B for additional 10 min. UV detection was performed at 310 nm.

Warfarin (both *R*- and *S*-warfarin) at 10 μg/mL in 0.05 M sodium hydroxide aqueous solution was used as an internal standard. Standards were prepared by spiking blank human plasma with *R*- and *S*-acenocoumarol and *R*- and *S*-phenprocoumon stock solutions. An aliquot of 1 mL of a plasma sample, control, or standard was spiked with 100 μL of internal standard, adjusted to pH 3.5 with 1 M hydrochloric acid, extracted with 5 mL of toluene for 20 min, and centrifuged at 1000 *g* for 5 min. The organic phase was collected, evaporated to dryness, reconstituted in 150 μL of eluent A, and assayed. Under these conditions, retention times of *S*-phenprocoumon, *R*-phenprocoumon, *R*-warfarin, *S*-warfarin, *R*-acenocoumarol, and *S*-acenocoumarol were 9.5, 11.5, 13.5, 17, 24, and 29 min, respectively.

Linear calibration curves for *R*- and *S*-acenocoumarol were constructed over the range from 15 to 2000 μg/L. Correlation coefficients were greater than 0.9998. Within-day (intraday) and between-day (interday) coefficients of variation were 4.6% and 7.8% for *R*-acenocoumarol and 4.7% and 6.1% for *S*-acenocoumarol, respectively. Average recoveries were better than 86.3% for *R*-acenocoumarol and 84.3% for *S*-acenocoumarol. The limits of detection and quantification were 5 and 15 μg/L, respectively, for both compounds. This assay was free of interference from the following drugs: alprazolam, amitriptyline, bupivacaine, carbamazepine, chlordiazepoxide, citalopram, clobazam, clomipramine, clonazepam, clozapine, desipramine, diazepam, diclofenac, diphenhydramine, doxepin, fentanyl, flunitrazepam, flupentixol, fluphenazine, flurazepam, fluvoxamine, haloperidol, imipramine, lamotrigine, levomepromazine, lidocaine, lorazepam, maprotiline, mefenamic acid, mepivacaine, mianserin, midazolam, nefazodone, nordazepam, nortriptyline, olanzapine, opipramol, oxcarbazepine, penfluridol, phenobarbital, pipamperone, promazine, quinine, ranitidine, sertraline, *S*-ibuprofen, sotalol, temazepam, thiopental, thioridazine, tolfenamic acid, topiramate, trazodone, triazolam, trimethoprim, trimipramine, venlafaxine, zolpidem, and zuclopenthixol. Carbamazepine coeluted with *S*-warfarin.

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ACETAMINOPHEN

CHEMISTRY

Acetaminophen is a synthetic nonopiate derivative of *p*-aminophenol. Its chemical name is *N*-(4-hydroxyphenyl) acetamide. Other names include Paracetamol, Tempra, and Tylenol. Its molecular formula is $C_8H_9NO_2$, with a molecular weight of 151.2 and a CAS number of 103-90-2. Acetaminophen is a white, crystalline powder with a slightly bitter taste. It is soluble in boiling water and freely soluble in alcohol. Acetaminophen has a pK_a of 9.51.

METHODS

Assay 1 Johnson and Plumb [1] compared HPLC with monolithic column and UPLC with UPLC column coupled with QTof mass spectrometer in the determination of metabolites of acetaminophen in human urine.

The Waters Acquity ultra-high-permformance liquid chromatography (UPLC) system was used. The stationary

phases were a Merck ChromSpeed monolithic column (50 × 4.6 mm) and a Waters Acquity column (50 × 2.1 mm, 1.7 μm). The column temperature was maintained at 40°C. Solvent A was 0.1% formic acid in water, and solvent B was acetonitrile. The mobile phase was delivered in a linear gradient of 0 to 40% B in 10 min. The flow rate was 500 μL/min for the UPLC column and 2 mL/min for the monolithic column, respectively.

The Waters QTof micro mass spectrometer was operated in positive-ion mode: nebulization gas (300 L/h, 250°C), cone gas (0 L/h), source temperature (120°C), capillary voltage (3200 V), cone voltage (30 V), collision gas (argon at 5.3×10^{-5} Torr), collision energies (5 and 25 eV), acquisition rate (0.3 s), and interscan delay (0.1 s). Leucine–enkephalin was used as the lock mass (m/z 556.2771) with a lockspray frequency of 5 s.

Urine was collected 1 h after two acetaminophen tablets (500 mg each) were taken orally by a volunteer and stored at −20°C. Before injection, urine samples were diluted 1 : 5 in distilled water. The injection volume was 10 μL. The column eluent was split to the mass spectrometer at 150 μL/min. Ion chromatograms were extracted at m/z 232 for acetaminophen sulfate, m/z 271 for acetaminophen-*S*-cysteine conjugate, m/z 328 for acetaminophen–glucuronide, and m/z = 427 for acetaminophen–glutathione conjugate, respectively. Retention times were 2.0 and 3.9 min for acetaminophen–glucuronide and acetaminophen–glutathione, respectively.

Assay 2 Makino et al. [2] described the simultaneous determination of ibuprofen, acetaminophen, indomethacin, and salicylic acid in human serum by capillary-zone electrophoresis and micellar electrokinetic chromatography. The Agilent HP^{3D} CE system equipped with a diode-array UV detector was utilized. The separation of drugs was carried out on an Agilent fused-silica extended light path capillary (64.5 cm × 50 μm internal diameter). The internal diameter of the so-called bubble cell capillary was 150 μm and the distance between the inlet end and the detector was 56 cm. The electrophoresis running buffer was 100 mM boric acid (pH 8.8), which was filtered through a 0.45-μm Millipore type HV filter and degassed before use. The fused-silica capillary was rinsed with running buffer for 4 min before each analysis. Injections were carried out by the vacuum system at 50 mmHg for 8 s. A constant voltage of 30 kV was applied at 25°C. UV detection was performed at 200 nm.

3-Isobutyl-1-methylxanthine at 50 μg/mL in acetonitrile was used as an internal standard. An aliquot of 100 μL of serum samples was mixed with 200 μL of the internal standard solution, vigorously mixed for 30 s, and centrifuged at 13400 *g* for 5 min. The supernatant was assayed. Under these conditions, migration times for acetaminophen, internal standard, indomethacin, ibuprofen, and salicylic acid were about 3.1, 3.5, 4.0, 4.4, and 5.4 min, respectively (estimated from the published electropherogram).

Calibration curves for acetaminophen were constructed in the therapeutic-to-toxic range with correlation coefficients greater than 0.999. The limit of detection was 4 μg/mL. Intraday and interday coefficients of variation of the migration time were 0.17–0.95% and 1.14–2.02%, respectively. Intraday and interday coefficients of variation of the relative peak area were 0.20–21.59% and 5.05–20.41%, respectively.

Assay 3 Baranowska et al. [3] developed an HPLC method for simultaneous determination of imipenem, paracetamol, dipyrone, vancomycin, amikacin, fluconazole, cefazolin, prednisolone, dexamethasone, furosemide, and ketoprfen in human urine. The Merck–Hitachi liquid chromatographic system consisting of a model L6200A "intelligent" pump, model L7480 diode-array detector, a model 7360 fluorescence detector, and a Rheodyne injector with a 20-μL loop was utilized. The stationary phase was a Merck LiChroCART Purospher C_{18}e analytical column (125 × 3 mm, 5 μm particle size) protected with a precolumn (4 × 4 mm, 5 μm particle size) of the same packing material. Solvent A was 0.05% trifluoroacetic acid in water; solvent B, methanol; and solvent C, acetonitrile. The mobile phase was delivered in a gradient mode as follows:

Time (min)	%A	%B	%C	Flow Rate (mL/min)
0	92	6	2	0.75
10	50	42	8	0.65
20	25	55	20	0.60
25	92	6	2	0.75

UV detections were performed at 300, 243, 259, 210, 210, 274, 242, 242, 234, and 254 nm for imipenem, paracetamol, dipyrone, vancomycin, fluconazole, cefazolin, prednisolone, dexamethasone, furosemide, and ketoprofen, respectively. Amikacin after derivatization was monitored by a fluorescence detector at an excitation wavelength of 355 nm and an emission wavelength of 415 nm. The runtime of an injection was 25 min. Under these conditions, retention times were 4.0, 4.9, 6.7, 8.1, 9.5, 10.0, 10.9, 13.3, 14.1, 16.0, and 19.0 min for imipenem, paracetamol, dipyrone, vancomycin, amikacin, fluconazole, cefazolin, prednisolone, dexamethasone, furosemide, and ketoprofen in human urine, respectively.

Stock solutions of these drugs at 1 mg/mL were separately prepared in water/methanol (50 : 50, vol/vol) and stored at −18°C. Working solutions containing these drugs were prepared by mixing individual stock solutions and diluting them with water/methanol (90 : 10, vol/vol). Standards were prepared by spiking drug-free human urine with working stock solutions. An aliquot of 0.75 mL of a urine sample or standard was adjusted to pH 8.0 with 1.5 M sodium hydroxide, mixed with 1.5 mL of acetonitrile and 1.5 mL of methanol, filled to the 10-mL mark with water, shaken for 1 min, and centrifuged at 22°C at 6500 rpm for 15 min. The supernatant was collected and assayed. The injection volume was 20 μL.

A calibration curve for paracetamol was constructed in the range of 0.5–45 μg/mL. The correlation coefficient was 0.9996. The recovery of paracetamol from urine ranged from 97.1% to 103.6%. The coefficient of variation for the assay was less than 6.9%. The accuracy in the relative percentage error was less than 3.6%. Limits of detection and quantification were 0.13 and 0.42 μg/mL, respectively.