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Simultaneous analysis of flunixin, naproxen, ethacrynic acid, indomethacin, phenylbutazone, mefenamic acid and thiosalicylic acid in plasma and urine by high-performance liquid chromatography and gas chromatography–mass spectrometry

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ABSTRACT

Simple and reproducible high-performance liquid chromatographic (HPLC) and gas chromatographic–mass spectrometric (GC–MS) methods have been developed for the simultaneous analysis of several acidic drugs in horse plasma and urine. Although the capillary GC–MS column provided better separation of the drugs than the reversed-phase C_8 (3 μm, 75 mm) HPLC column, the total analysis time with HPLC was shorter than the total analysis time with GC–MS. The HPLC system equipped with a diode-array detector provided simultaneous screening (limit of detection 100–500 ng/ml) and confirmation (limit 1.0 μg/ml) of the drugs. The HPLC system equipped with fixed-wavelength ultraviolet and fluorescence detectors provided a relatively sensitive screening [limit of detection 50–150 ng/ml for ultraviolet and 10 ng/ml for fluorescence (naproxen only) detectors] of the drugs. However, the positive samples had to be confirmed by using either the diode-array detector or the GC–MS system. The GC–MS system provided simultaneous screening and confirmation of the drugs at very low concentrations (20–50 ng/ml).

INTRODUCTION

Naproxen (6-methoxy-α-methyl-2-naphthaleneacetic acid), flunixin (3-pyridine-carboxylic acid 2-[[2-methyl-3-trifluormethyl]phenyl]amine), indomethacin ([1-(chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid), phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione) and mefenamic acid (N-(2,3-xylyl)-anthranilic acid) are analgesic and anti-inflammatory agents that have been approved for use in horses and other animals. Phenylbutazone is very potent in relieving pain, reducing fever and diminishing swelling due to inflammatory disorders [1]. Several studies have shown that flunixin can be used effectively in the management of endotoxin-induced cardiovascular damage in horses [2,3]. Another acid drug, ethacrynic acid, is commonly used as a diuretic agent in animals. Controlled use of phenylbutazone, where the plasma phenylbutazone levels

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should not exceed 3–5 μg/ml, is allowed in racehorses by several racing jurisdictions in the USA and other countries. However, recent trends indicate that phenylbutazone is often used simultaneously with other analgesic drugs, which exacerbates the problem of analgesic abuse in horses.

Phenylbutazone is commonly quantitated by high-performance liquid chromatography (HPLC) with UV detection [4–7]. Although several sensitive methods are available for the analysis of other drugs or their metabolites (naproxen by HPLC [8–10] and gas chromatography (GC) [11,12], indomethacin by HPLC [2] and GC [13], and ethacrynic acid by GC–MS [14]), a simple and reproducible method for the simultaneous analysis of the combination of acidic drugs in urine and plasma is necessary for controlling the illegitimate use of these drugs in animals. Recently, Hardee *et al.* [9] have developed an HPLC method for the simultaneous analysis of several analgesic drugs in horse plasma, but their method lacks sensitivity an confirmation capability. Therefore, the aim of this study was to develop a simple method for the simultaneous screening and confirmation of several acidic drugs by HPLC and GC–MS.

EXPERIMENTAL

Reagents

Standards of naproxen, flunixin, indomethacin, phenylbutazone, mefenamic acid and ethacrynic acid were obtained from Sigma (St. Louis, MO USA). HPLC-grade extraction solvents were obtained from Fisher (Minneapolis, MN, USA). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, USA).

Instruments

The HPLC systems used in this study were a Hewlett Packard 1990 liquid chromatograph equipped with a diode-array detector (HP-1040A), and a Beckman Gold (120 pumps and 166 UV detector) equipped with a Spectrovision FD-300 fluorescence detector, a Spectra Physics 8825 autosampler and chrome jet integrator. The GC–MS analysis was performed with an HP 5980C mass spectrometer and an HP 5880 gas chromatograph.

Sample preparation

Plasma. A 1-ml plasma sample containing 0.1, 0.5, 1.0, 2, 2.5, 5.0 and 10.0 μg of various drugs was mixed with 1 ml of 0.1 *M* hydrochloric acid and 10 ml of dichloromethane. The mixture was rotoracked for 10 min and centrifuged (1500 *g*) for 15 min, and the aqueous phase was aspirated from the surface. The organic layer was collected into another tube and dried at 45°C under a stream of nitrogen. For the HPLC analysis, the dried residue was redissolved in the mobile phase. For the GC–MS analysis, the dried residue was derivatized by mixing with 10 μl of BSTFA before analysis. Mefenamic acid or indomethacin was used as the internal standard for the quantitative analysis.

Urine. For HPLC analysis, 50 μl of control or NaOH-treated urine were mixed with 1.0 ml of the mobile phase, and 20 μl of the mixture were direclty injected into the column. For GC–MS, 1–2 ml of urine (control or NaOH-hydrolysed) were acidified to pH 3 with saturated phosphate buffer and extracted with 10 ml of dichloromethane. The dichloromethane layer was separated by centrifugation at 10 000 *g*, and dried at 45°C under nitrogen. The dried residue was derivatized by adding 10 μl of BSTFA to the sample, and 1 μl of the derivatized residue was injected into the GC–MS system.

Determination of the UV spectra

Chromatograms obtained from each sample were stored in an HP-85B personal computer containing the Data Evaluation Pack-1 program. The UV spectrum of each drug was determined by recalling the peak of interest and entering the retention time and peak parameters in the computer program. The UV spectrum obtained from the sample and from the standard were compared by printing the spectra together for superimposition.

Chromatographic conditions

HPLC. The mobile phase was 0.05 *M* phosphoric acid–acetonitrile (55:45, v/v). The column was a Supelcosil LC-8 (7.5 cm × 4.6 mm I.D., 3 μm particle size). The flow-rate was 1.0 ml/min. The UV absorbance was measured at 235 nm. For fluorescence detection, the excitation wavelength was 235 nm and the emission wavelength was 405 nm. The diode-array detector was set to scan wavelengths from 209 to 402 nm at a bandwidth of 4 nm. The sampling frequency was set at 80 ms.

GC–MS. The GC–MS analysis was performed by using an Econocap capillary column, SE-54 (30 m × 0.25 mm I.D.). The oven temperature was programmed at 20°C/min from an initial temperature of 150°C to a final temperature of 280°C; the run time was 15 min. The injector temperature was 250°C and the injection mode was splitless. For selected-ion monitoring (SIM), three ions were selected for each drug (Table I).

Calibration, recovery and precision

For plasma samples, calibration was done by adding known amounts of various acidic drugs and the internal standard to plasma (50 μl of 500 ng/ml solution) and by extracting the samples as described above. For HPLC analysis, the dried residue was redissolved in the mobile phase and 20 μl were injected. For GC–MS analysis, each sample was derivatized with 10 μl of BSTFA, and 1.0 μl was injected into the GC–MS system. Standard curves were prepared by plotting the concentration against the peak-height ratio of the standard to the internal standard. The standard curves were analysed with a linear regression program. The overall recovery was determined by adding known amounts (10 ng/ml to 100 μg/ml) of drugs in ethyl acetate and in plasma or urine, analysing the unextracted

TABLE I

MAJOR IONS PRODUCED BY THE ELECTRON-IMPACT IONIZATION OF VARIOUS ACIDIC DRUGS AND THE THREE IONS SELECTED FOR THE SELECTED-ION MONITORING

Drug	Major ions [*m/z* (relative abundance)]	Ion selected for SIM (*m/z*)
Naproxen	185(100), 243(90), 302(30)[a], 73(30), 279(15)	185, 248, 302
Flunixin	353(100), 263(95), 251(40), 209(10), 368(15)[a]	209, 163, 353
Indomethacin	139(100), 141(20), 111(10), 158(5), 246(3), 312(5), 429(5)[a]	139, 312, 429
Mefenamic acid	223(100), 313(70)[a], 208(40), 180(5), 195(3)	223, 313, 208
Ethacrynic acid	73(100), 243(50), 319(40), 339(20), 374(15)	243, 319, 374
Phenylbutazone	73(100) 246(60), 218(28), 337(20), 380(20)[a]	246, 218, 380
Thiosalicylic acid	225(100), 73(80), 136(20), 226(15), 283(3)	225, 136, 226

[a] Molecular ion.

drug and the extracted sample by HPLC and GC–MS, and comparing the peak-height ratio of the unextracted drug with that of the extracted drug. The concentrations of a drug calculated experimentally were compared with the concentrations added. The precision was determined as described by Van Loenhout *et al.* [8]. Correlation coefficients were calculated as described by Lin *et al.* [15].

For urine samples, calibration was done by adding known amounts of the drugs to 1.0 ml of urine and analysing 20 μl of urine as described earlier. Recovery and precision were determined as described for plasma.

RESULTS AND DISCUSSION

Chromatographic separation

In regulatory drug testing, acidic drugs are commonly screened by a thin-layer chromatography (TLC) method [16] in which urine samples are extracted, spotted on a TLC plate, developed in an appropriate solvent and sprayed with Mandelin reagent [17]. Although the TLC method provides broad screening of acidic drugs, it lacks sensitivity and cannot be used for quantitation, and the TLC plates may contain interfering spots. [16].

Several investigators have developed HPLC methods for the quantitative screening of acidic drugs, using various mobile phases, columns and extraction procedures for different drugs [7–12, 18–20]. This study indicated that the reversed-phase C_8 (3 μm, 75 mm) HPLC and the capillary SE-54 GC columns provided clear separation of several acidic drugs present in plasma or urine samples. The retention times of naproxen, flunixin, thiosalicylic acid, ethacrynic acid, indomethacin, phenylbutazone and mefenamic acid were 3.2, 2.8, 2.2, 4.0, 5.0, 6.8 and 8.0 min, respectively, for the HPLC column (Figs. 1 and 2), and 6.8, 7.2, 11.4, 8.4, 13.4, 10.2 and 7.4 min, respectively, for the GC column (Fig. 3). The naprox-

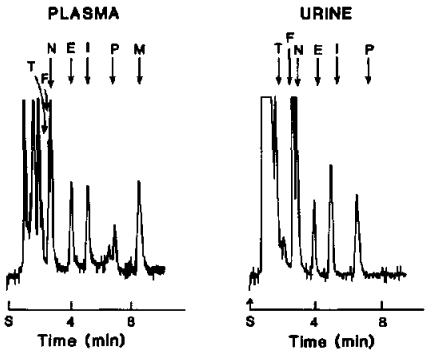

Fig. 1. Chromatograms of plasma and urine samples containing 500 ng/ml each of thiosalicylic acid (T), flunixin (F), naproxen (N), ethacrynic acid (E), indomethacin (I), phenylbutazone (P) and mefenamic acid (M) obtained with a C_8 (3 μm, 75 mm) column and a diode-array detector. The drugs were monitored at 235 nm.

en and flunixin peaks were resolved well by the GC column, but not by the HPLC column, especially at high concentrations. However, naproxen and flunixin peaks were distinghuised by the fluorescence detector because of naproxen's strong fluorescence properties (Fig. 2) [21].

Sensitivity and linearity of the standard curves

Table II lists the linear regression parameters for the peak-height values. This study indicated that (1) the single-wavelength UV detector was more sensitive than the diode-array detector, (2) both the UV and the diode-array detectors were relatively more sensitive for naproxen than for phenylbutazone or thiosalicylic acid, (3) the sensitivities of different acidic drugs were comparable when determined by the GC–MS method, and (4) the fluorescence detector was most sensitive for the detection of naproxen in plasma or urine (Table II). Since the UV absorption spectra of the drugs in acidic solution (Figs. 4 and 5) show differences in the maximum absorbance, and since the UV detectors were set at a fixed wavelength of 235 nm, optimal conditions may not have been achieved for certain drugs. The sensitivity of the GC–MS method was comparable with the sensitivity reported by Sioufi *et al.* [22] for the GC analysis of phenylbutazone and greater than the sensitivities reported by Gyllenhaal and Albinsson [23] and Budd [24] for phenylbutazone or mefenamic acid.

Recovery

The overall recovery and precision for each drug are shown in Table III. The recovery of each drug from plasma was *ca.* 95%, and the assay demonstrated good precision. The accuracy of the assay was best at the 10.0 μg/ml level. The limit of detection of the GC–MS and the fluorescence (for naproxen only) meth-

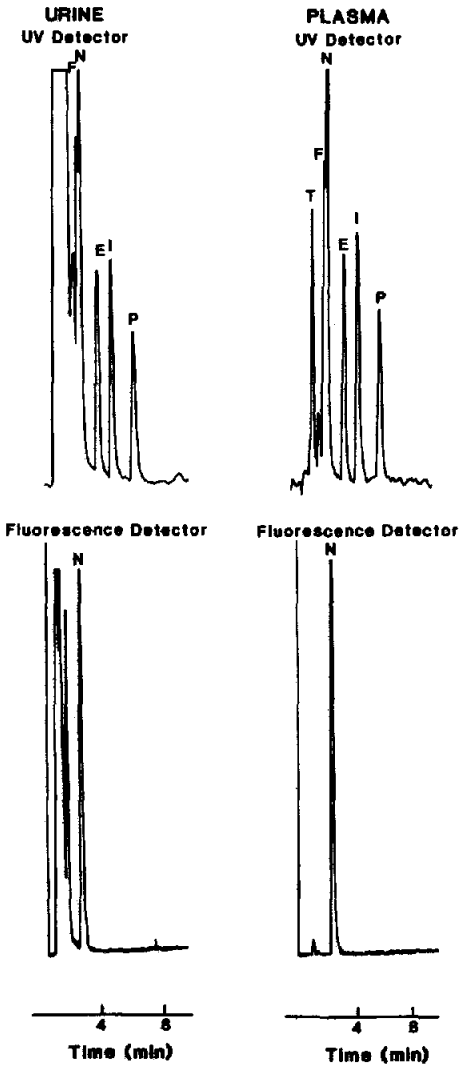

Fig. 2. Chromatograms of plasma and urine samples containing 500 ng/ml each of various acidic drugs obtained with fluorescence and UV detectors and a C_8 (3 μm, 35 mm) column. The UV wavelength was set at 235 nm; for fluorescence detection, the excitation wavelength was 235 nm and the emission wavelength was 405 nm.

ods was 20–50 ng/ml when 1.0 ml plasma or 20 μl of urine (for fluorescence detection only) was used. However, the limit of detection for the HPLC–UV and the HPLC–diode-array detection methods was *ca.* 50–250 ng/ml when 1.0 ml plasma or 20 μl of urine was used. To screen plasma or urine samples containing low levels of these drugs, 5–10 ml of the sample can be extracted, pooled, concen-

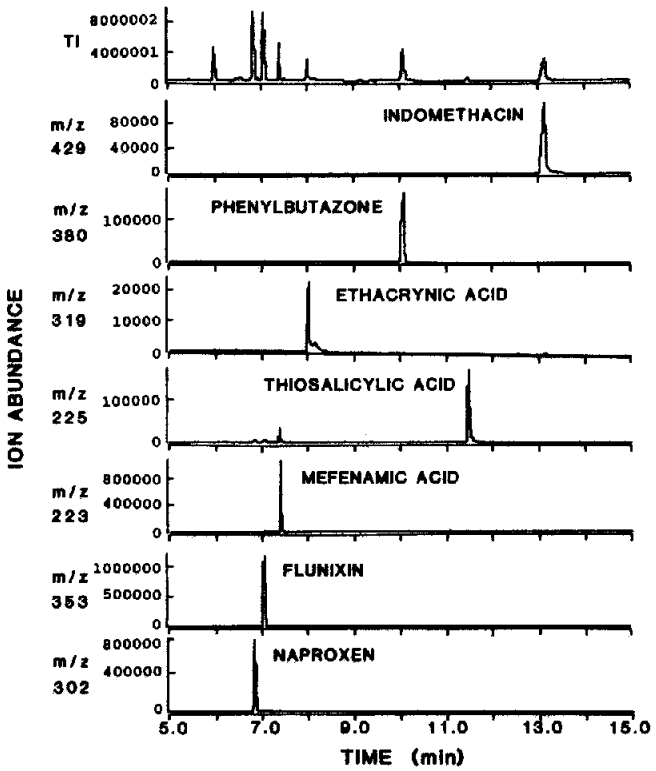

Fig. 3. GC–MS of a urine sample containing 200 ng/ml each of various acidic drugs. Individual drugs are identified by the major ion produced by the drug. The analytical procedure is described in the text.

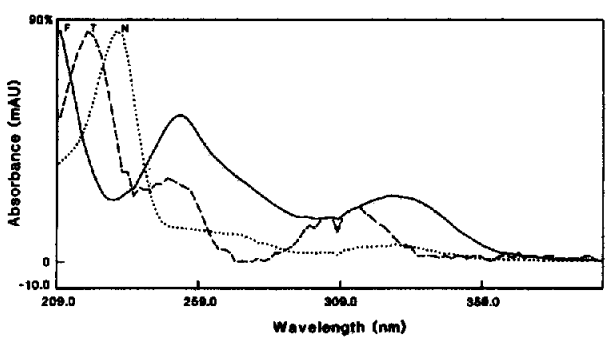

Fig. 4. UV absorption curves for thiosalicylic acid (T), flunixin (F) and naproxen (N).

TABLE II

LINEAR REGRESSION PARAMETERS FOR THE STANDARD CURVES FOR EACH DRUG PRESENT IN PLASMA AND URINE

EA = ethacrynic acid; MA = mefenamic acid, PBZ = phenylbutazone; TSA = thiosalicylic acid; $n = 6$.

	Naproxen	Flunixin	EA	Indomethacin	MA	PBZ	TSA
Plasma							
HPLC–UV							
Slope	1.2 ± 0.08	0.5 ± 0.05	0.3 ± 0.02	0.3 ± 0.01	0.3 ± 0.02	0.2 ± 0.02	0.1 ± 0.02
r	0.9990	0.9995	0.9995	0.9993	0.9990	0.9850	0.9695
HPLC–F							
Slope	2.0 ± 0.05	–	–	–	–	–	–
r	0.9998	–	–	–	–	–	–
HPLC–DAD[a]							
Slope	0.8 ± 0.02	0.4 ± 0.02	0.3 ± 0.01	0.25 ± 0.01	0.25 ± 0.04	0.3 ± 0.05	0.08 ± 0.02
r	0.9998	0.9995	0.9990	0.9995	0.9992	0.9895	0.9690
GC–MS							
Slope	1.25 ± 0.05	1.16 ± 0.04	1.00 ± 0.05	1.00 ± 0.02	0.90 ± 0.05	0.95 ± 0.04	0.85 ± 0.07
r	0.9995	0.9990	0.9997	0.9995	0.9990	0.9993	0.9970
Urine							
HPLC–UV							
Slope	1.4 ± 0.07	0.8 ± 0.1	0.53 ± 0.07	0.45 ± 0.03	0.30 ± 0.02	0.36 ± 0.02	0.20 ± 0.03
r	0.9945	0.9905	0.9995	0.9990	0.9993	0.9995	0.9890
HPLC–F							
Slope	2.3–0.1	–	–	–	–	–	–
r	0.9895	–	–	–	–	–	–
HPLC–DAD[a]							
Slope	0.9 ± 0.03	0.5 ± 0.07	0.5 ± 0.03	0.5 ± 0.04	0.3 ± 0.05	0.3 ± 0.06	0.1 ± 0.07
r	0.9995	0.9994	0.9969	0.9990	0.9983	0.9805	0.9690

[a] Diode-array detection.

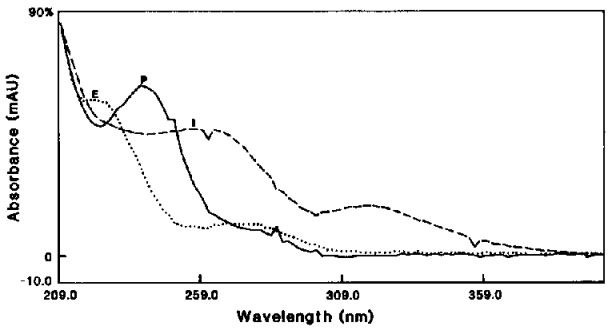

Fig. 5. UV absorption curves for ethacrynic acid (E), phenylbutazone (P) and indomethacin (I).

trated and analysed by GC–MS in SIM mode. However, concentrated samples may contain interfering peaks when analysed by HPLC.

Drug confirmation

Once the presence of a drug has been detected by TLC, HPLC or GC–MS, it must be confirmed by GC–MS [25] or by the diode-array detector [26]. Identification of samples by GC–MS in SIM mode can be simultaneously confirmed by comparing the sample's mass spectrum with that of the standard mass spectrum, using the following criteria: (1) the retention time of the drug peak in the sample is within 1.0% of the standard retention time, (2) at least three ions are used for confirmation; (3) the abundance of each ion is within 20% of the abundance of each ion present in the standard. Similarly, samples screened by the diode-array detector can also be confirmed by comparing the UV absorption patterns of the standard and the sample, as shown in Fig. 6. This study indicated that a drug concentration of at least 1–3 μg/ml was needed to achieve a good quality UV absorption curve. Since urine samples cannot be concentrated before HPLC anal-

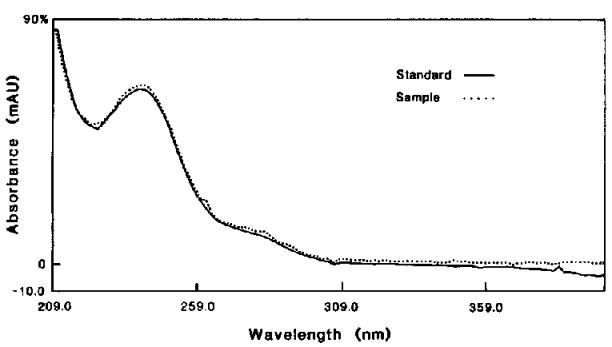

Fig. 6. Comparison of the UV absorption curves of phenylbutazone present in a plasma sample and standard phenylbutazone.

TABLE III

OVERALL PRECISION OF THE HPLC AND GC–MS METHODS BY SERIAL ANALYSIS OF PLASMA SAMPLES

Values is parentheses are coefficients of variation (%).

Drug	Method	Amount recovered (ng/ml or μg/ml)			
		50 ng/ml added	1.0 μg/ml added	100 μg/ml added	100 μg/ml added
Naproxen	HPLC–UV	–	1.2 ± 0.03 (2.6)	9.8 ± 0.15 (1.5)	100.9 ± 3.0 (3.1)
	HPLC–F[a]	50.1 ± 2.0 (4.0)	1.03 ± 0.01 (0.98)	11 ± 0.11 (1.2)	98 ± 2.7 (3.0)
	HPLC–DAD[b]	–	1.11 ± 0.04 (3.8)	10.7 ± 0.13 (1.3)	110 ± 3.2 (3.0)
	GC–MS	48 ± 1.5 (3.12)	1.02 ± 0.04 (4.3)	10.3 ± 0.11 (1.1)	103 ± 2.9 (2.9)
Flunixin	HPLC–UV	–	1.11 ± 0.05 (4.5)	10.1 ± 0.11 (1.2)	101 ± 2.9 (3.0)
	HPLC–DAD	–	1.13 ± 0.03 (2.9)	10.7 ± 0.19 (1.9)	98.6 ± 3.1 (3.2)
	GC–MS	49 ± 2.3 (4.64)	1.11 ± 0.02 (2.1)	10.9 ± 0.20 (1.9)	100.9 ± 2.9 (3.2)
Ethacrynic acid	HPLC–UV	–	1.3 ± 0.05 (4.1)	11.0 ± 0.13 (1.2)	101 ± 3.2 (3.0)
	HPLC–DAD	–	1.2 ± 0.03 (2.9)	10.2 ± 0.11 (1.1)	98 ± 4.7 (4.9)
	GC–MS	53 ± 6.0 (11.3)	0.96 ± 0.05 (5.2)	10.7 ± 0.2 (1.9)	107 ± 5 (4.9)
Indomethacin	HPLC–UV	–	0.98 ± 0.02 (2.1)	10.7 ± 0.15 (1.5)	98.6 ± 3.1 (3.3)
	HPLC–DAD	–	1.03 ± 0.01 (1.0)	10.6 ± 0.11 (1.0)	101 ± 4.2 (4.2)
	GC–MS	47 ± 2 (4.2)	1.01 ± 0.03 (3.0)	9.8 ± 0.10 (1.1)	97.2 ± 2.9 (3.1)
Mefenamic acid	HPLC–UV	–	1.03 ± 0.02 (2.0)	11.2 ± 0.13 (1.2)	110 ± 3.1 (3.0)
	HPLC–DAD	–	0.96 ± 0.04 (4.5)	10.9 ± 0.15 (1.4)	106 ± 3.7 (3.5)
	GC–MS	49 ± 1.3 (2.6)	1.03 ± 0.04 (3.9)	11.0 ± 0.10 (0.95)	101 ± 4.3 (4.3)
Phenylbutazone	HPLC–UV	–	0.95 ± 0.05 (5.0)	10.6 ± 0.15 (1.5)	97 ± 2.7 (2.9)
	HPLC–DAD	–	1.1 ± 0.03 (2.9)	12.0 ± 0.13 (1.1)	98 ± 3.6 (3.9)
	GC–MS	63 ± 1.2 (2.5)	1.2 ± 0.02 (1.9)	11.3 ± 0.11 (0.99)	103 ± 4.2 (4.1)
Thiosalicylic acid	HPLC–UV	–	1.3 ± 0.06 (4.9)	13.2 ± 0.32 (2.4)	11 ± 11.6 (10.5)
	HPLC–DAD	–	0.98 ± 0.05 (5.1)	11.7 ± 0.30 (2.8)	113 ± 10.2 (10.0)
	GC–MS	51 ± 2.0 (4.0)	1.1 ± 0.02 (2.0)	10.3 ± 0.11 (1.1)	98 ± 3.6 (3.9)

[a] Fluorescence detection.

[b] Diode-array detection.

ysis, the concentration of drugs in the sample may be the limiting factor in drug confirmation with the diode-array detector. Therefore, samples containing low levels of the drugs can be confirmed by concentrating the sample ten- to twenty-fold, and analysing the concentrated sample by GC–MS in the SIM mode.

In conclusion, plasma or urine can be screened for acidic drugs by using an HPLC system equipped with a fixed-wavelegth UV detector. Positive samples can be confirmed by either HPLC with a diode-array detector or GC–MS. If the amount of the drug in plasma or urine is expected to be above the detection limit of the diode-array detector, both screening and confirmation can be achieved by using the HPLC with a diode–array detector. Because of the time and cost involved, it may not be possible to screen large numbers of samples by GC–MS.

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