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Short Communication

Simultaneous determination of flumazenil, midazolam and metabolites in human biological fluids by liquid chromatography

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ABSTRACT

A simple procedure for the simultaneous determination of flumazenil, midazolam, 1-hydroxymethylmidazolam and 4-hydroxymidazolam in plasma or urine in surgical patients is described. The assay involves a preliminary extraction of the drugs, metabolites and internal standard (flurazepam) from biological fluid into an organic solvent mixture (dichloromethane–diethyl ether, 40:60 v/v). The extract was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was re-dissolved in distilled methanol (80 μl) and a 30-μl aliquot was injected via an automatic sampler into the liquid chromatograph and eluted with the mobile phase (32% acetonitrile in 0.004 *M* sodium hydrogenphosphate buffer containing 1 ml of triethylamine and adjusted to pH 7.2) at a flow-rate of 1.5 ml/min on a 30-μm C_8 precolumn linked to a 4-μm Nova-pak C_{18} cartridge column (100 mm × 8 mm I.D.) at ambient temperature (25°C). The eluate was detected at 220 nm.

INTRODUCTION

Midazolam, a water-soluble benzodiazepine widely used in anaesthesia and for sedation of artificially ventilated patients in intensive care units, is predominantly metabolized to 1-hydroxymethylmidazolam and 4-hydroxymidazolam, which are subsequently conjugated to form glucuronides [1]. Flumazenil, a benzodiazepine antagonist, has been used for the reversal of benzodiazepine-induced anaesthesia, sedation and intoxication [2]. Various chromatographic techniques are reported for the measurement of midazolam on its own or simultaneously with its metabolites. These include gas chromatography (GC) with electron-capture detection [3–5], high-performance liquid chromatography (HPLC) with UV detection [6,7], GC with mass spectroscopic (MS) detection [8,9] and radioimmunoassay [10]. A GC method was reported for the assay of flumazenil

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[11]. Recently a comparison of HPLC, GC and GC–MS was made for the determination of total and unbound midazolam in human plasma [12]. It was concluded that GC with nitrogen-selective detection was recommended for routine plasma assay with sensitivity of 20 ng/ml, HPLC was less sensitive (100 ng/ml) and GC–MS was suggested for analysis validation.

We have been interested in monitoring the pharmacodynamics [13] and pharmacokinetics of both midazolam and flumazenil when the latter was used to reverse the action of the former drug in anaesthesized patients. This paper reports the development of a simple assay which measured simultaneously midazolam, its two hydroxy metabolites and their glucuronides, and flumazenil in plasma and urine samples.

EXPERIMENTAL

Analytical standards

The hydrochloride salts of midazolam, 1-hydroxymethylmidazolam, 4-hydroxymidazolam, flumazenil, desmethylflumazenil and flurazepam (internal standard) were of pharmaceutical purity and obtained from Roche Far East Research Foundation (Hong Kong).

Reagents

All reagents were of analytical grade and included dichloromethane, diethyl ether, methanol and triethylamine (Merck, Darmstadt, Germany) which were freshly glass-distilled before use. Sodium phosphate buffer (0.1 *M*, pH 9) for extraction was prepared by dissolving sodium hydrogenphosphate in water with adjustment of pH to 9 using sodium hydroxide. Deionized water was doubly glass-distilled, and acetonitrile was of HPLC grade (Mallinckrodt, Paris, KY, USA).

Apparatus and chromatographic conditions

The chromatographic system consisted of a Waters M6000A reciprocating piston pump, a Waters Model 7100B intelligent sample processor (WISP TM) and a Model 994 programmable photodiode-array detector (Waters Assoc., Milford, MA, USA). Analysis was performed on a C_{18} reversed-phase cartridge column (Nova Pak cartridge, 4 μm, 100 mm × 8 mm I.D.) linked to a C_8 pre-column (30 μm, 30 mm × 4.6 mm I.D.). The isocratic mobile phase used was a mixture of 32% (v/v) acetonitrile in 0.04 *M* sodium hydrogenphosphate buffer with 1 ml/l triethylamine and a resulting pH at 7.2. This was degassed by vacuum before use, and the flow-rate was 1.5 ml/min. The detector was set at 220 nm. The WISP autoinjector was programmed to run for 40 min per sample, using 10% methanol in distilled water as the rinse solvent, at ambient temperature (25 ± 1°C). Other apparatus used included 10 ml centrifuge tubes with PTFE-lined screw-caps and 15 ml glass tubes with tapered base of 100 μl capacity. All glassware was treated with silanizing agent as described previously [14] to avoid loss of drugs due to adsorption to glass surface.

Preparation of standard solutions and calibration samples

Stock solutions of flumazenil, desmethylflumazenil, midazolam and its two hydroxy metabolites were prepared by dissolving accurately weighed quantities in distilled methanol to give a concentration of 1.0 mg/ml. These stock solutions were used to spike the drug-free plasma (obtained from a blood bank) or urine to cover various calibration ranges: flumazenil (4–200 ng/ml), midazolam (20–1000 ng/ml), 1-hydroxymethylmidazolam (10–500 ng/ml) and 4-hydroxymidazolam (10–500 ng/ml). Fresh calibration external standards and extraction processed standards (containing 200 ng/ml of each compound) of the plasma or urine standards were run together with twenty unknown samples as a batch processed by the automatic sampler, which was operated normally overnight. Two standards were placed at the beginning and at the end of the batch of unknowns. This was used to check the linearity and reproducibility of the chromatographic system.

Sample preparation

Plasma or urine after deglucuronidation treatment (0.5 ml) was pipetted accurately into a 10-ml centrifuge tube with a tightly fitting screw-cap (with PTFE septum), and 50 μl of methanolic

standard solution containing 200 ng of flurazepam were added as internal standard. After the addition of 1 ml of 0.1 *M* sodium hydrogenphosphate buffer (pH 9) and 4 ml of organic solvent mixture (dichloromethane–diethyl ether, 60:40, v/v) the whole content was extracted by shaking for 15 min using an automatic shaker at 45 rpm. The emulsion was separated by centrifugation for 10 min at 1870 *g* using a bench centrifuge at 10°C. The organic extract was transferred to a glass tube with a tapered base and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in distilled methanol (80 μl), and an aliquot (30 μl) was injected into the HPLC system via the automatic sampler. A batch of 24 samples, including two external standards and two extraction-processed standards, which were placed one at the beginning and one at the end of the batch, and twenty plasma or urine unknown samples, was run by the automatic sampler usually from 6 p.m. onward till the next morning. The temperature throughout was 25 ± 1°C. No loss of accuracy was noted.

Deglucuronidation of urine samples

To a 0.25-ml urine sample, 0.75 ml of acetate buffer (pH 5.4) and 500 U of β-glucuronidase were added in a 10-ml centrifuge tube. The tube, with a PTFE-lined screw-cap, was incubated at 37°C for 18 h. The mixture was centrifuged after the addition of 20 μl of 5 *M* sodium hydroxide solution to inactivate the enzyme and 5 ml of organic solvent mixture for extraction. The extract was treated as described in *Sample preparation*.

Stability studies

Flumazenil, midazolam and metabolites were introduced into drug-free plasma or urine at a final concentration of 200 ng/ml. The samples were divided into portions of 0.5 ml in 10 ml centrifugation tubes. The stability of the drugs in biological fluids was tested by measuring their concentrations at 4°C overnight and at −20°C for six months.

Quantitation

Calibration graphs were constructed by spiking flumazenil, midazolam and their metabolites into drug-free plasma samples. Diluted stock methanolic solutions were prepared to cover concentration ranges 4–200 ng/ml for flumazenil, 10–500 ng/ml for 4-hydroxymidazolam and 1-hydroxymethylmidazolam and 20–1000 ng/ml for midazolam. Between-day standards of concentration 200 ng/ml were determined to obtain the between-batch variation of the assays. A batch of 24 samples was run by the automatic sampler usually from 6 p.m. onward till the next morning. Two external methanolic standards and two extraction-processed standards (one plasma and one urine sample) were placed one at the beginning and one at the end of a batch of twenty unknown samples. The calibration graphs were repeated six times for measurement of reproducibility. The recoveries of the drugs and metabolites were determined for each compound by comparing their peak heights from extracted plasma samples with that of the internal standard in the methanolic solution added after extraction. Extraction efficiency was determined by peak-height ratios for each compound to the non-extracted internal standard. Comparison was made between samples that were spiked before and after extraction.

RESULTS AND DISCUSSION

Performance of the HPLC system

The analytical peaks of flumazenil, 4-hydroxymidazolam, 1-hydroxymethylmidazolam, flurazepam, internal standard and midazolam were well resolved (Figs. 1 and 2) and the retention times were 3.8, 10.5, 13.6, 18.5 and 33.5 min, respectively. No endogenous peak from blank plasma, urine or enzyme-treated urine samples was observed which might interfere with the peak-height measurement of the analytical peaks. During the selection of the mobile phase, the pharmacologically inactive metabolites, desmethylflumazenil and the flumazenil carboxylic acid metabolite, were included for screening. Desmethylflumazenil could be resolved if a lower flow-rate

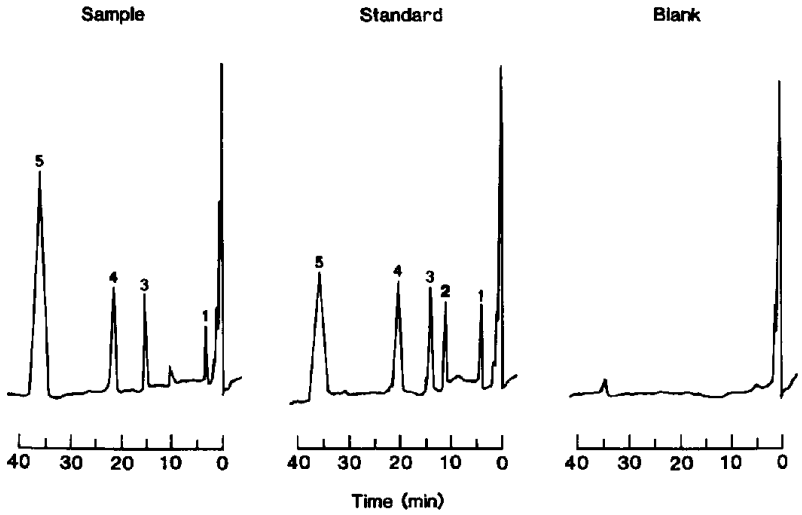

Fig. 1. Chromatograms of plasma extracts of a patient sample (left: 1 = flumazenil, 28 ng/ml; 3 = 1-hydroxymethylmidazolam, 243 ng/ml; 4 = flurazepam (I.S.), 200 ng/ml; 5 = midazolam, 1076 ng/ml), a calibration standard (middle: 1 = flumazenil, 48 ng/ml; 2 = 4-hydroxymidazolam, 63 ng/ml; 3 = 1-hydroxymethylmidazolam, 251 ng/ml; 5 = midazolam, 538 ng/ml), and a drug-free sample (right).

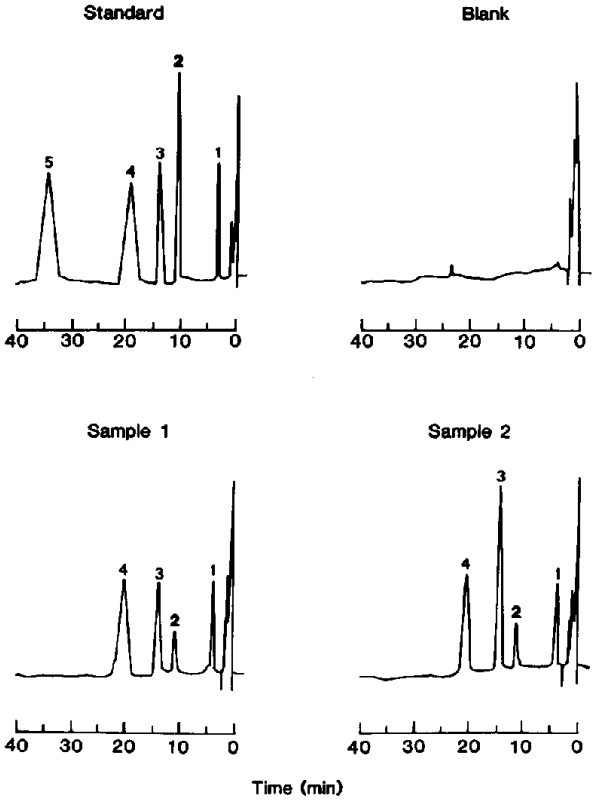

Fig. 2. Chromatograms of urine extracts of a calibration standard (top left: 1 = flumazenil, 53 ng/ml; 2 = 4-hydroxymidazolam, 501 ng/ml; 3 = 1-hydroxymethylmidazolam, 248 ng/ml; 4 = flurazepam (I.S.), 200 ng/ml; 5 = midazolam, 539 ng/ml), a urine blank (top left), a patient sample (bottom left: 1 = 48 ng/ml; 2 = 25 ng/ml; 3 = 184 ng/ml) and the same patient sample after deglucuronidation (bottom left: 1 = 46 ng/ml; 2 = 23 ng/ml; 3 = 378 ng/ml).

of mobile phase or a lower percentage of acetonitrile in the mobile phase was used whilst the carboxylic acid metabolite did not appear in the chromatogram over a period of 2 h after injection of the extract. Using the present chromatographic conditions, the desmethylflumazenil peak was masked by the solvent front of the chromatogram. None of the other drugs administered before, during and after anaesthetic procedure to the paediatric patients, including alfentanil, atropine, bupivacaine, lignocaine and neostigmine interfered with the assay.

Recovery and stability of drugs processed by the assay

In previous study [5], *n*-hexane was used as the extraction solvent with an extraction recovery of midazolam from plasma samples of 90.3 ± 2.0% (mean ± S.D.) in a range 90–800 ng/ml. Dichloromethane–diethyl ether (2:3) was chosen because it could be evaporated at a low temperature in a shorter period (lower boiling point) to avoid loss due to instability during the concentration stage. The mean (range) recoveries of flumazenil, midazolam, 4-hydroxymidazolam, 1-hydroxymethylmidazolam and flurazepam (internal standard) were 92.0% (86–94%), 90.5% (85–96%), 80.0% (78–83%), 82.0% (70–85%) and 93.6% (89–

TABLE I

CALIBRATION AND PRECISION OF THE HPLC ASSAY USING SPIKED PLASMA ($n = 3$) AND URINE ($n - 3$) SAMPLES

Flumazenil			4-OH Midazolam			1-OH Midazolam			Midazolam		
Spiked concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Spiked concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Spiked concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Spiked concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	C.V. (%)
4	3.89 ± 0.25	6.68	10	9.10 ± 0.38	4.19	10	9.28 ± 0.47	5.06	20	19.85 ± 1.55	7.89
10	9.56 ± 0.46	4.81	25	24.82 ± 1.50	6.04	25	24.71 ± 1.53	6.19	50	49.48 ± 4.05	8.16
25	24.64 ± 1.31	5.32	62.5	63.70 ± 2.02	3.17	62.5	63.28 ± 2.83	4.48	125	123.71 ± 6.43	5.20
50	52.92 ± 2.37	4.48	125	121.68 ± 3.44	2.83	125	124.87 ± 5.88	4.71	250	251.55 ± 15.21	6.05
100	102.20 ± 4.70	4.60	250	259.72 ± 11.84	4.56	250	252.38 ± 14.34	5.68	500	506.19 ± 23.49	4.64
200	200.09 ± 6.34	3.17	500	499.93 ± 31.04	6.21	500	503.21 ± 24.86	4.94	1000	1005.15 ± 61.51	6.12
Batch standards ($n = 8$)[a]											
200	204.11 ± 9.84	4.82	200	211.68 ± 9.40	4.44	200	209.36 ± 10.45	4.99	200	216.47 ± 14.50	6.70
Calibration graph	$y = 0.00436 + 0.0189x$		$y = 0.0127 + 0.0097x$			$y = 0.0191 + 0.0061x$			$y = 0.010 + 0.00194x$		
Correlation coefficient	$r^2 = 0.9998$		$r^2 - 0.9997$			$r^2 = 0.9997$			$r^2 = 0.9987$		

[a] Plasma or urine standards processed in a batch of 24 samples as a measure of between-day variation.

96%). Recently, a solid-phase extraction using C_{18} cartridges was reported with recoveries in excess of 90% for midazolam and its metabolites using 1 ml of plasma [15]. The present method is more economical with accepted recovery results. Similar extraction recoveries of flumazenil and midazolam were reported by Vletter *et al.* [16] using the same 60:40 mixture of diethyl ether and dichloromethane. The limit for detection was 4 ng/ml for flumazenil and 10 ng/ml for midazolam and metabolites, yielding a detector response approximately equal to three times the detector noise. This compares favourably with the reported 50 ng/ml from the solid-phase extraction method [17]. The drugs were stable in plasma or urine samples, and no significant change of drug concentrations after storage for one day at 4°C and at −20°C for up to six months occurred. No observable alteration in concentration was detected when the concentrates were processed overnight by the automatic injector.

Accuracy, reproducibility and calibration of the assay

The calibration of peak-height ratios *versus* drug concentrations in plasma samples was linear for the four compounds with acceptable coefficients of linear regression (Table I). The between-day coefficients of variation of 200 ng/ml for all compounds were satisfactory in either urine and plasma standards. Ideally, two different concentrations should be employed, one at the low concentration end. The 200-ng/ml level was chosen as the mid-range for three compounds, but this was the highest concentration for flumazenil calibration.

Application

The assay was used to monitor simultaneously the plasma concentrations of flumazenil and midazolam and the urine concentrations of the drugs and metabolites in twelve paediatric patients undergoing circumcision surgery who were given midazolam (0.5 mg/kg orally as premedication and 0.5 mg/kg intravenously during anaesthetic induction) and flumazenil (10 μg/kg intravenously and 5 μg/kg/min as intravenous infusion until the patient woke up. The mean (±S.D.) pharmacokinetic parameters, as determined by statistical moment analysis, including elimination half-life, total body clearance and volume of distribution at steady state, were 35.3 ± 13.8 h, 20.69 ± 6.91 ml/min/kg and 1.0 ± 0.2 l/kg for flumazenil, and 101.7 ± 31.3 h, 15.4 ± 3.2 ml/min/kg and 1.9 ± 0.6 l/kg for midazolam. Details of this study were published elsewhere [17]. Analysis of the urinary data indicated that no unchanged midazolam was recovered in the urine of the ten out of twelve children from whom urine collection was successful. A wide variation in the recovery, as a percentage of dose, of the hydroxy metabolites and their glucuronides of midazolam was observed: 0–0.23% as 1-hydroxymethylmidazolam, 0.64–13.2% as 1-hydroxymethylmidazolam glucuronide, 0.03–1.12% as 4-hydroxymidazolam and 0.01–1.15% as 4-hydroxymidazolam glucuronide. One child, A.K.K., was an extensive hydroxylator. Unchanged flumazenil, 5.84–13.8% of the dose administered, was recovered in all urine samples. Desmethylflumazenil was detected but was not measurable by the HPLC method as its analytical peak was too close to the solvent front of the chromatogram. The extensive hydroxylater child also excreted the largest amount of unchanged flumazenil. More study on the metabolism of flumazenil and midazolam in children and adults is needed to substantiate the findings. An improvement of the HPLC assay could be developed to include the measurement of desmethylflumazenil by using a solvent gradient programme for the delivery of mobile phase.

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